Universidade de Aveiro Departamento de Biologia
2007

João André da Mota Pedrosa

AVALIAÇÃO DAS COMPONENTES AMBIENTAL E FISIOLÓGICA DA INGESTÃO EM CLADÓCEROS

ASSESSMENT OF THE ENVIRONMENTAL AND PHYSIOLOGICAL COMPONENTS OF FEEDING

Universidade de Aveiro Departamento de Biologia
2007

João André da Mota Pedrosa

AVALIAÇÃO DAS COMPONENTES AMBIENTAL E FISIOLÓGICA DA INGESTÃO EM CLADÓCEROS

ASSESSMENT OF THE ENVIRONMENTAL AND PHYSIOLOGICAL COMPONENTS OF FEEDING IN CLADOCERANS

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia e Ecotoxicologia, realizada sob a orientação científica do Professor Doutor António Nogueira, Professor (Professor Associado com Agregação) do Departamento de Biologia da Universidade de Aveiro

o júri

presidente

Prof. Dr. Amadeu Mortágua Velho da Maia Soares

professor catedrático da Universidade de Aveiro

Prof. Dr. Lúcia Maria Candeias Guilhermino

professor catedrática do ICBAS da Universidade do Porto

Prof. Dr. António José Arsénia Nogueira (orientador)

professor associado com agregação da Universidade de Aveiro

agradecimentos

Nenhuma obra nasce de geração espontânea. Em regra, resulta da acumulação de trabalho de muita gente, de que o autor é face visível.
O trabalho desenvolvido ao longo dos últimos meses teve como resultado final a dissertação de Mestrado. Aproveito, desde já, para agradecer às pessoas que directa ou indirectamente permitiram que este trabalho tenha sido possível de realizar.
Em primeiro lugar e, naturalmente… surge a minha família. Pai, mãe e irmã (Ana) agradeço a vossa paciência, ajuda e força nestes já 24 anos de caminhada! Para vocês e… por muitas palavras que aqui usasse nunca seria possível dizer tudo o que sinto. Por isso… apenas um enorme e eterno: "Obrigado"! Avós, avô, tios, tias, primos e primas, sem distinção, também não estão esquecidos!

Em segundo lugar, o meu honroso agradecimento ao meu professor e orientador, o Prof. Dr. António Nogueira que é o grande responsável e incentivador pelos primeiros passos nesta tão complexa e fascinante área denominada Ecotoxicologia! Ao longo deste já ano e meio em que me dá a honra de trabalhar consigo, espero corresponder às expectativas e confiança que tem depositado em mim. Desde o início do trabalho sempre se colocou à minha disposição, aconselhou, explicou e demonstrou os caminhos a seguir. Ao incansável professor o meu grande, grande obrigado!

Uma outra pessoa muito importante nesta caminhada foi a colega e amiga Fátima, que me acolheu desde que me encontrava a concluir a licenciatura. Com a Fátima passei muitas horas nesses laboratórios do Departamento de Biologia. Muitas foram espectaculares; já outras…mais concretamente aquelas em que os relógios assinalavam "horas da madrugada" confesso que as trocava por outras fora do Departamento! Se existe alguma palavra que rime com Fátima essa será certamente trabalho, ou melhor: trabalho com boa disposção!

Também deixo aqui umas palavras de agradecimento aos meus colegas de laboratório. Desde o nosso cantor Henrique, passando pelos Mestres Miguel e Abel, Marco (Jaime), Pestana, Sizenando, Raquel, Sara, Bruno e Filipa e Joaquim a todos vós o meu "muito obrigado"! Queria também agradecer aos projectistas e mestrandos do laboratório. Todos juntos criámos uma grande equipa!
Subindo um pouco pelo Departamento acima… queria também agradecer às meninas do andar de cima: Susana Loureiro, Mónica e Inês!
Gostava também de agradecer aos colegas dos Laboratórios do lado: Ramiro, em especial, sem esquecer a Marta, a Salomé, a Guida, o Bruno, a Sara, a Catarina e a Joana.

Para o fim, e não menos importantes, reservei um espaço para os meus amigos de curso e Universidade. Não querendo individualizar, sob pena de não ter espaço para escrever o nome de todos… aqui vos deixo um forte abraço! Sempre convosco!

À Fundação para a Ciência e Tecnologia agradeço o financiamento deste estudo através da atribuição de uma bolsa de iniciação à investigação científica (POCI/AMB/55801/2004)

palavras-chave **Toxicidade, Zinco, Cobre, Daphnia magna, Modelo do Ligando Biótico, Especiação, Composição Química Ambiental, Alimento, Ingestão de alimento, Temperatura**

Resumo

Actividades antropogénicas variadas, tais como exploração de minas, processamento ou uso de metais e/ou substâncias que contêm poluentes metálicos são amplamente reconhecidas como fontes de contaminantes induzindo efeitos letais e não letais nos organismos de água doce. Apesar da existência de políticas ambientais protectoras para critérios de qualidade da água (CQA) contra metais pesados, a avaliação de tal contaminação tem falhado. Contrariamente ao pequeno número de características usadas na regulamentação ambiental, a toxicidade dos metais é o resultado de interacções complexas entre as características da água e a especiação dos metais.

Neste contexto, os objectivos deste trabalho são: determinar os principais factores ambientais que afectam a toxicidade aguda e crónica dos metais pesados cobre e zinco; e desenvolver um modelo que preveja as taxas de ingestão do cladócero *Daphnia magna* Straus ao longo do seu ciclo de vida perante águas com diferentes características físicas e químicas – o efeito do aumento de temperatura, dureza, alcalinidade, pH e concentração de alimento será testado.

Este estudo identificou que em situações de exposição aguda, os iões responsáveis pela dureza, cálcio e magnésio, juntamente com o sódio, podem diminuir a letalidade de peixes e crustáceos. O aumento da concentração destes iões resulta na diminuição da letalidade devido à competição com os iões metálicos livres, removendo-os dos sítios activos dos organismos. Contudo, em exposições crónicas de cobre, o aumento da concentração destes iões não resulta em efeitos protectores adicionais nos cladóceros.

O aumento de pH e alcalinidade resulta, também, em diminuições da toxicidade para os organismos devido a implicações directas na especiação do metal, levando à redução da concentração do ião metálico livre, a espécie metálica mais tóxica. A nível agudo, para pH muito ácidos (pH = 5), a toxicidade pode novamente ser reduzida, devido ao efeito competitivo do ião hidrogénio. Valores baixos ou elevados de temperatura podem acentuar o efeito tóxico dos metais.

A matéria orgânica pode desempenhar, também, um papel protectivo. O aumento da concentração da matéria orgânica dissolvida faz diminiur a toxicidade dos metais, embora a sua fonte tenha implicações no efeito protectivo. Adicionalmente, a fracção particulada da água pode reduzir ou aumentar a toxicidade dos metais.

Depois do levantamento das principais características da água que afectam a toxicidade dos organismos, levámos a cabo uma série de experiências onde características físicas e químicas da água foram variadas. Os tradicionais testes agudos e crónicos não revelam o modo de acção de um tóxico, uma vez que a toxicidade para os organismos pode resultar de reduções na ingestão de alimento e/ou de aumento dos custos metabólicos. Para além deste factor, a influência das características físicas e químicas nos organismos não tem sido focada em testes de toxicidade e o seu possível efeito tóxico não está bem documentado. Deste modo, é deveras importante medir as as taxas de ingestão dos organismos em situações de ausência de metais a fim de obter determinações mais correctas acerca do efeito dos metais. Os ensaios realizados com *Daphnia magna* Straus, clone F, sugeriram que, em situações de ausência de metal, a composição química da água não afecta significativamente a ingestão dos organismos. Contudo, as dáfnias responderam aos níveis de alimento, e a quantidade de alimento ingerido aumentou de forma alométrica de acordo com o tamanho do organismo. Este aumento estará relacionado com o aumento dos custos metabólicos do organismo à medida que o seu corpo vai aumentando de tamanho. Os organismos também responderam às variações de temperatura testadas de 20º até 30º C. A 30º C os organismos ingeriram menor quantidade de alimento, quando comparado com os organismos testados a 20º e a 25º C. A pequena quantidade de alimento ingerida, juntamente com os custos metabólicos elevados resulta em menores ciclos de vida de *D. magna*. Os ensaios, adicionalmente, sugeriram que a temperatura óptima dos organismos deverá rondar os 24º C, valor esse que está de acordo com estudos anteriormente realizados por outros autores.

keywords **Toxicity, Zinc, Copper, Daphnia magna, Biotic Ligand Model, Metal speciation, Environmental Chemical Composition, Food, Feeding ingestion, Temperature**

Abstract

Various anthropogenic activities, such as mining, processing or use of metals and/or substances that contain metal pollutants are widely recognized as important contaminants inducing lethal and sub-lethal effects to freshwater organisms. Although the existence of protecting environmental policies to water quality criteria (WQC) against heavy metals, the evaluation of contamination has failed. Contrarily to the few water characteristics used in environmental policies, metal toxicity, in freshwaters, is the result of complex interactions between water characteristics and metal species.

In this context, this work has the following objectives: determine the main factors affecting the acute and chronic toxicity of the heavy metals copper and zinc; and develop a model predicting the feeding rates of the cladoceran *Daphnia magna* Straus according to the body length under a wide range of physical and chemical water characteristics – the effects of increased temperature, hardness, alkalinity, pH and food concentration will be tested.

This study identified that at an acute level the competitive effect of the hardness ions, calcium and magnesium, as well as sodium ions can diminish the lethality in fish and crustaceans. The increased concentration of these ions results in decreased lethality due to competition with the free metals ions at the active sites of the organisms, even being poor competitors to the active sites. At a chronic level, however, the competitive effects of these cations seem not to be so important in protecting cladocerans against copper toxicity and, hence, increased concentrations do not result in additional protection to the organisms.

Increases in pH and alkalinity of the waters result in decreased toxicity to the organisms due to direct implications in metal speciation reducing the concentration of the free metal ion – the most toxic metal species. A decrease in pH to 5 results in protective effects at acute level possibly due to competing effects of the hydrogen ion. Temperature variations for low and high values can induce the metal toxicity.

The organic matter content can, also, play a protective role. Increasing the concentration of dissolved organic matter results in lower toxicity to organisms. However, its source is important in the protective effect against metals. Additionally, the particulate fraction can increase or reduce the metal toxicity.

After this focus about the most important characteristics affecting organisms' toxicity, we conducted a series of experiments where physical and chemical water characteristics were varied. The traditional acute and chronic tests do not reveal of how does a toxicant act. Toxicity to organisms can result from reductions in food intake and/or increased metabolic costs. Moreover, water physical and chemical characteristics have not been determined individually in risk assessments, and their toxic effect to organisms is not well known. The importance of measuring the feeding ingestion of the organisms in free toxic situations is, hence, of major importance in correct risk assessments of metals. Our feeding experiments conducted with the cladoceran Daphnia magna Straus, clone F, suggested that, in free-metal situations, the chemical composition of the water is of lower importance. However, daphnids respond to food levels, and the amount of ingested food increases in an allometric way according to the body length of the organism. This increase can possibly be related to increased metabolic costs of the organism with body increases. The organisms also responded to temperature variations from 20º to 30º C. At 30º C the organisms ingested few amount of food when compared to the individuals tested at 20º and 25º C. The little amount of ingested food addicted to higher metabolic costs result in shorter life cycles of D. magna. The conducted assays, additionally, suggested that the optimal temperature of the organisms will be reached at ~24º C, which is in accordance with reported literature.

«Não é o Homem, são os homens que habitam este planeta.
A pluralidade é a lei da Terra»
Hannah Arendt

INDICE

List of Tables

List of Figures

List of Equations

1. General Introduction

The environmental contamination is characterized by a great diversity of pollutants that affect the water quality and constitute an eminent intoxication risk to humans. Heavy metals are continuously released in aquatic systems as a result of natural (weathering of soils and rocks, volcanic eruptions) and anthropogenic (activities related to mining, processing or use of metals and/or substances that contain metal pollutants) sources and have long been recognized as important pollutants (Jones et al., 2003). Water quality criteria (WQC) have been applied by regulator entities in order to determine toxicity effects to aquatic biota. However, it has been recognized by both researchers and regulators that the performed WQC tests in laboratory did not correctly predict the toxicity in natural waters. Due to the complex influence of multiple natural factors occurring in natural waters, such as pH, hardness and alkalinity (Chakoumakos et al., 1979) it has been difficult to regulate metal toxicity in natural waters (Breault et al., 1996).

During the last years some efforts/corrections were done by the regulators aiming to determine heavy metal toxicity in a more reliable way. Firstly, it was recognized that the total metal criteria approach would not correctly predict toxicity and, hence, only the dissolved heavy metal fraction was used to determine toxicity. Later, the competitive effect of calcium ions was included in toxicity assessments, via a calculated hardness-based adjustment (USEPA, 1993). However, regulators recognized that such consideration would not be enough to incorporate metal's bioavailability to aquatic organisms (Reiley, 2007) because the effects of DOC, alkalinity and pH were not considered. Ultimately it was introduced water effect ratios (WERs) into WQC in the environmental policy of the United States of America in order to account with metal bioavailability and toxicity to aquatic biota (USEPA, 1994). Metal toxicity endpoints (e. g. LC_{50} concentration) are calculated in both receiving and laboratory water samples. Since the receiving water LC50 is typically higher than the laboratory test LC_{50}, the WQC is multiplied by the WER (ratio of the LC_{50} in the receiving water to the LC_{50} in the laboratory water) to adjust the WQC to site-specific water quality characteristics. This

experimental design is usually repeated three times to account for seasonal variations in water quality characteristics. The big disadvantages of this method are time-consuming and costly (Reiley, 2007).

In this context, the biotic ligand model (BLM) approach has recently gained increased interest among the scientific and regulatory communities because of its potential to predict metal toxicity to aquatic waters over multiple ambient water quality, considering the three Cs of metal toxicity: concentration, competition, and complexation (Di Toro et al., 2001). The BLM approach permits the rational explanation of the effect of variation in water chemistry on the toxicity of metals to aquatic organisms (Allen, 2002). Historically, the BLM concept evolved from the ancestors FIAM (Free Ion Activity Model) (Morel, 1983) and the GSIM (Gill Surface Interaction Model) (Pagenkopf, 1983). The FIAM predicted that biological effects were due to the binding of the free hydrated metal ion, rather than total metal, to a finite number of aniotic sites on the gills in competition with protective cations. The GSIM additionally recognized that inorganic anions (it is recognized the importance of DOM but it is not included) would complex metals, reducing their availability and accepted that other metal species in addition to the free cation might bind to the critical sites.

At the moment, the environmental regulatory policies of US Environmental Protection Agency, has just introduced the BLM to predict copper toxicity and a number of other metals-BLM criteria are under development (zinc, nickel, silver and lead) (Reiley, 2007). In European Union context, however, the regulatory policies of environmental risk assessment, till this moment, have not introduced it. However, the Water Framework Directive-WFD (Directive 2000/60/EC of the European Parliament) alerted to the need of obtaining rapid and significant improvements in the ecological quality of surface waters. Such improvements can be obtained using the Biotic Ligand Model.

The conceptual framework of the BLM has a strong physiological basis because it considers that metal's toxicity occur due to the binding of the free metal ions at the physiologically active sites of action or biotic ligands (*e.g.*, fish gills) of the aquatic organism. The biotic ligand is defined as the part of a living organism

that is capable of regulating its physiological and metabolic functions in response to environmental perturbations (Niyogi and Wood, 2003). These active sites are suggested to be specific proteins involved in the active uptake of essential ions across the gills. The active sites are characterized by: the conditional binding constants (log K), that calculates the binding strength to a certain ligand – higher log K values mean stronger linkage between the active sites and the ligands while lower log k values mean weak binding strength; and the maximum number of binding sites (B_{max}) or binding site density (Playle, 1998). Once the strength of metal binding to a biological membrane is determined, competitive effects of cations can be assessed, producing equilibrium stability constants, such as Ca-gill and H-gill interactions, as well as DOM-gill interactions. However, for such determinations it is assumed that rates of metal-gill interactions are fast and gill surfaces have a finite number of metal binding sites (Pagenkopf, 1983). It has been suggested that various species of fish can share a single gill-metal conditional stability constant, possibly due to equal fundamental gill membrane characteristics. Differences in toxicity among fish species have been attributed, in part, to differences in physiological repair, compensation, distribution, and elimination mechanisms rather than to major differences in metal adsorption and thus metal bioavailability (MacRae et al., 1999; Taylor et al., 2003).

The BLM, hence, relates environmental ligands that bind cationic metals (dissolved organic matter, sulfide, thiosulfate, chloride carbonate and hydroxide) and the biotic ligand (gill surface in fish) which also binds cationic metals (figure 1.1). The model assumes that water chemistry variables such as competing cations (e. g. Na^+, Ca^{2+}, Mg^{2+}, and H^+), inorganic ligands (e. g. hydroxides, chlorides, carbonates) and organic ligands (it only considers the dissolved organic fraction, excluding the particulate organic fraction) play a crucial role in the bioavailability of the free metal ion and, consequently, metal's toxic effects (Di Toro et al., 2001; Niyogi and Wood, 2003). It is presupposed that is the uncomplexed cationic species of a metal which interacts at the biotic ligands (Paquin et al., 2000).

The interaction of the biotic and abiotic (organic and inorganic) components on the bioavailability of metals to aquatic organisms is considered by inserting the

biotic ligand (e. g. gills) into powerful aquatic chemistry programs (e. g. Windermere humic aqueous model – WHAM (Tipping, 1998)), which run using thermodynamic equilibrium stability constants and account the activity coefficients (or chemical speciation) of several ions present in solution (Playle, 1998).

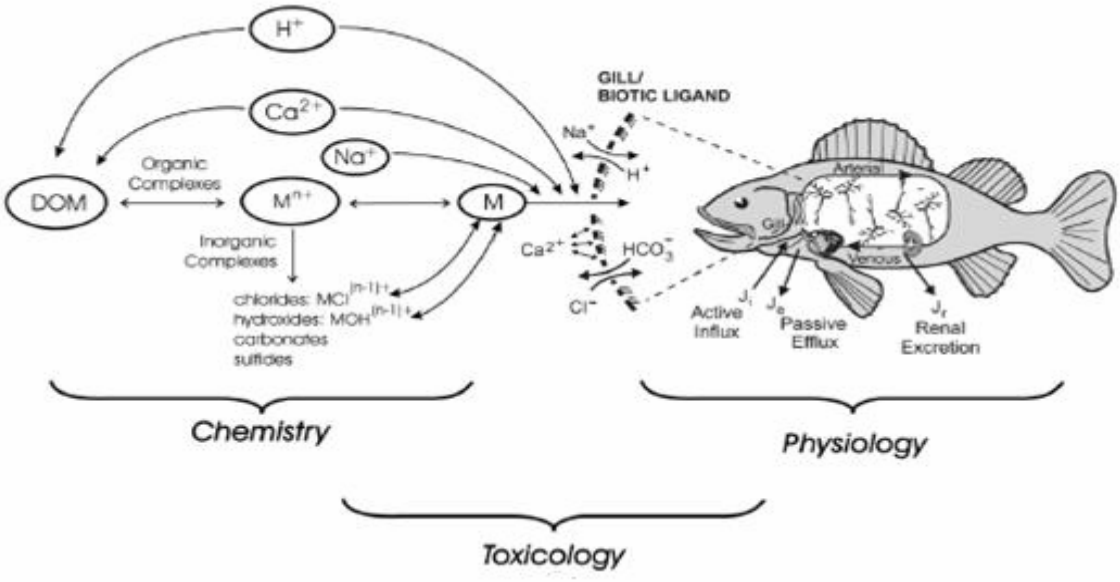

Figure 1.1– Schematic presentation of the BLM approach. Toxicity is a result of the interaction between the chemical and the biological components occurring in the aquatic environment (Paquin et al., 2002).

The BLM concept was originally applied to predict lethality in fish species, important organisms in freshwater ecosystems that accumulate great amounts of persistent pollutants (Playle et al., 1993). Recently, the BLM research has focused in its adaptation to aquatic invertebrates, such as the crustacean *D. magna* (de Schamphelaere and Janssen, 2002). As a consequence of the small size and the frequency of molt in juvenile daphnids it is difficult to readily sample and analyze the gills, possible sites of toxic action. In this approach, metal binding constants are derived exclusively from toxicity data (de Schamphelaere and Janssen, 2002), using the same fundamental concept of the BLM that mortality occurs when metal-binding at the site of action reaches a critical level. The Biotic Ligands are

considered as hypothetical ligands consisting of all external metal interaction sites determining metal toxicity (Heijerick et al., 2002).

Another point of increased attention is the recalibration of the acute model to include also chronic predictions, along the organisms' life cycle (de Schamphelaere et al., 2005). A simplified approach would be the calibration of the acute BLM into a chronic BLM by lowering the critical heavy metal concentration. However, such adjustment cannot be simply done by lowering the concentrations of the heavy metals. The effects that affect acute toxicity can be of different relative importance at a chronic level (de Schamphelaere et al., 2005). Additional problem in predicting chronic effects of heavy metal exposures can result from the addition of food. In acute toxicity tests, food is not added but in chronic tests it is, which can result in an additional metal complexing ligand. Consequently, increased food levels can result in decreased labile metal concentration and increased nutritional condition of the test organism (Hauri and Horne, 2004).

1.1. Objectives and structure of the thesis

This work arises in the sequence of the development of a Biotic Ligand Model (BLM) for copper and zinc, which objective is to evaluate the toxic response of freshwater organisms exposed to zinc or copper over a wide range of physic-chemical water characteristics to predict lethal and non lethal toxic effects in freshwater organisms. In this context, in first instance, we investigated and identified the major environmental water characteristics influencing metal acute and chronic toxicity. After identifying the major water parameters that can provoke great variations in toxicity to aquatic organisms, we conducted feeding experiments, using the cladoceran *Daphnia magna* Straus, along its life history without metal exposition. Experimental media ranged in hardness (from soft to very hard water) and alkalinity (from low to very high), which, consequently, resulted in little pH variations. These experiments were conducted at temperatures varying from standard (20º C) to very high (30º C). The results were, then, adjusted to a general predictive model for individual feeding rates per hour, which included the significant factors affecting feeding ingestion. The feeding rates of the

organisms consist in a particularly important endpoint determined to understand how a certain stressor affect organisms. Possible chronic toxicity, expressed at several parameters of the organism such as growth, fecundity or behavior, can be a result of feeding inhibition. The identification of this endpoint can be important in understanding why the enumerated chronic effects occur when an organism is exposed to a certain stressor. Our BLM approach consisted in ranging physic and chemical freshwater characteristics, without heavy metal addition. The aim was to understand how the above mentioned water characteristics can, by themselves, consist in additional stress to aquatic organisms.

Hence, the general objectives of this study were:

- Determine the major environmental factors affecting waterborne copper and zinc toxicity in reference freshwater organisms (e. g. fish and crustaceans) under acute and chronic heavy metal exposures
- Evaluate how do certain water physic-chemical characteristics interfere with *Daphnia magna* feeding ingestion;
- Determine possible factors that, traditionally, have not been included in the Biotic Ligand Model approaches but are important in determining copper and zinc toxicity;
- Develop a model predicting organisms' feeding rates over water characteristics including the significant interactions between water physic and chemistry to *D. magna* under different food levels.

In order to successfully reach the general proposed objectives, this thesis was structured as follows:

- The first chapter corresponds to the general introduction, where a brief explanation about the Biotic Ligand Model is given. The concept of BLM and the respective assumptions are described; the model is contextualized in the actual risk assessment policies

- The second chapter entitled **"Zinc and copper toxicity in aquatic organisms: relation between lethal and non lethal effects"**, describes how do the physic and chemical freshwater characteristics influence zinc and copper

toxicity. In this chapter, studies from several researchers were reported on this chapter. The influence of hardness, pH, major cation content (calcium, magnesium, potassium and sodium separately), alkalinity, temperature and dissolved and particulate organic matter were discussed for both fish and crustaceans, common reference organisms used in ecotoxicological assays.

- The third chapter, **"Evaluation of physic-chemical media characteristics in *Daphnia magna* Straus feeding rates"**, aims to develop a predictive model for feeding ingestions of *Daphnia magna* Straus under a variety of water characteristics. This chapter consists in the experimental assays. The feeding rates of *D. magna* Straus will be measured along daphnids' life cycle exposed to different media consisting in independent variations of hardness and alkalinity. Daphnids will be tested under a range of temperature (feeding rates will be tested at 20º, 25º and 30º C). Data will be statistically tested and the significant effects of each parameter and the interactions between them on the feeding rates of the organisms will be incorporated into the predictive model.

- The fourth chapter is dedicated to the final discussion and conclusions,

1.2. References

Allen, H.E., 2002. The biotic ligand model addresses effects of water chemistry on metal toxicity. International Council of Mining and Metals 7.

Breault, R.F., Colman, J.A., Aiken, G.R., McKnight, D., 1996. Copper Speciation and Binding by Organic Matter in Copper-Contaminated Streamwater. Environ. Sci. Technol. 30, 3477-3486.

Chakoumakos, C., Russo, R.C., Thurston, R.V., 1979. Toxicity of copper to cutthroat trout (*Salmo clarki*) under different conditions of alkalinity, pH and hardness. Environ. Sci. Technol. 13, 213-219.

de Schamphelaere, K.A.C., Janssen, C.R., 2002. A Biotic Ligand Model Predicting Acute Copper Toxicity for *Daphnia magna*: The Effects of Calcium, Magnesium, Sodium, Potassium, and pH. Environ. Sci. Technol. 36, 48-54.

de Schamphelaere, K.A.C., Lofts, S., Janssen, C.R., 2005. Bioavailability models for predicting acute and chronic toxicity of zinc to algae, daphnids, and fish in natural surface waters. Environ. Toxicol. Chem. 24, 1190-1197.

Di Toro, D.M., Allen, H.E., Bergman, H.L., Meyer, J.S., Paquin, P.R., Santore, R.C., 2001. Biotic ligand model of the acute toxicity of metals. 1. Technical basis. Environ. Toxicol. Chem. 20, 2383-2396.

Hauri, J.F., Horne, A.J., 2004. Reduction in labile copper in the 7-day *Ceriodaphnia dubia* toxicity test due to the interaction with zooplankton food. Chemosphere 56, 717-723.

Heijerick, D.G., De Schamphelaere, K.A.C., Janssen, C.R., 2002. Prediting acute zinc toxicity for Daphnia magna as a function of key water chemistry characterisitics: development and validation of a biotic ligand model. Environ. Toxicol. Chem. 21, 1309-1315.

Jones, B.G., Chenhall, B.E., Debertsion, F., Hutton, A.C., 2003. Geochemical comparisons between estuaries with non-industrialised and industrialised catchments: the Huon and Derwent River estuaries; Tasmania. Aust. J. Earth. Sci. 50, 653-667.

MacRae, R.K., Smith, D.E., Swoboda-Colberg, N., Meyer, J.S., Bergman, H.L., 1999. Copper binding affinity of rainbow trout (*Oncorhynchus mykiss*) and brook trout (Salvelinus fontinalis) gills: implications for assessing bioavailable metal. Environ. Toxicol. Chem. 18, 1180-1189.

Morel, F.M., 1983. Complexation: Trace metals and microorganisms. Principles of Aquatic Chemistry. Wiley Interscience, New York, pp. 301-308.

Niyogi, S., Wood, C.M., 2003. Effects of chronic waterborne and dietary metal exposures on gill metal-binding: Implications for the Biotic Ligand Model. Hum. Ecol. Risk. Assess. 9, 813-846.

Pagenkopf, G.K., 1983. Gill surface interaction model for trace-metal toxicity to fishes: role of complexation, pH, and water hardness. Environ. Sci. Technol. 17, 342-347.

Paquin, P.R., Gorsuch, J.W., Apte, S., Batley, G.E., Bowles, K.C., Campbell, P.G.C., Delos, C.G., Di Toro, D.M., Dwyer, R.L., Galvez, F., Gensemer, R.W., Goss, G.G., Hogstrand, C., Janssen, C.R., McGeer, J.C., Naddy, R.B., Playle, R.C., Santore, R.C., Schneider, U., Stubblefield, W.A., Wood, C.M., Wu, K.B., 2002. The biotic ligand model: a historical overview. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 133, 3-35.

Paquin, P.R., Santore, R.C., Wu, K.B., Kavvadas, C.D., Di Toro, D.M., 2000. The biotic ligand model: a model of the acute toxicity of metals to aquatic life. Environ. Sci. Policy 3, 175-182.

Playle, R.C., 1998. Modelling metal interactions at fish gills. Sci. Total. Environ. 219, 147-163.

Playle, R.C., Dixon, D.G., Burnison, K., 1993. Copper and cadmium-binding to fish gills - estimate of metal gill stability-constants and modeling of metal accumulation. Can. J. Fish. Aquat. Sci. 50, 2678-2687.

Reiley, M.C., 2007. Science, policy, and trends of metals risk assessment at EPA: How understanding metals bioavailability has changed metals risk assessment at US EPA. Aquat. Toxicol. 84, 292-298.

Taylor, L.N., Wood, C.M., Mcdonald, D.G., 2003. An evolution of sodium loss and gill metal binding properties in rainbow trout and yellow perch to explain species differences in copper tolerance. Environ. Toxicol. Chem. 22, 2159-2166.

Tipping, E., 1998. Humic Ion-Binding Model VI: An Improved Description of the Interactions of Protons and Metal Ions with Humic Substances. Aquatic Geochemistry 4, 3-47.

USEPA, 1993. Science Advisory Board. Notification of Consultation on Revisions to the Aquatic LifeWater Quality Criteria Guidelines. EPA-SABEPEC-CON-93-006.

USEPA, 1994. Water quality Standard Handbook. United States Environmental Protection Agency. Office of Water, Washington, DC.

2. Zinc and copper toxicity in aquatic organisms: relation between lethal and non lethal effects

João A. M. Pedrosa, Fátima T. Jesus, António J.A. Nogueira
CESAM & Departamento de Biologia, Universidade de Aveiro, Portugal
(To be submitted to Chemosphere)

Abstract

Copper and zinc are common contaminants in water bodies. However, water quality characteristics have been identified to vary the toxic effects from several folds in aquatic biota. In this paper, we describe ecotoxicological data of zinc and copper from a number of studies conducted with both fish and crustaceans at an acute and chronic level. Dissolved organic matter (DOM) concentration was observed to be the most protective effect against copper toxicity at acute and chronic levels, while the same protective effect against zinc toxicity is only observed at higher DOM concentration. The DOM source has also been identified as of major importance to the protective effect, especially for copper toxicity. Also the particulate fraction of the organic matter can reduce the toxic effect of both metals. Important competitive effects of major ions (Ca, Mg, Na, and H) for the biotic ligands were observed to copper and zinc at an acute level. However, at chronic concentrations contradictory results were obtained by researchers. pH and alkalinity can also play an important role in metal speciation, reducing the bioavailable free ion metal and, hence, the toxicity of the metal. Finally, once the majority of aquatic organisms are ectotherms, they have an optima range of temperature. Outside these limits, the toxic effect of metals is increased by the temperature effect.

Keywords: Copper, Zinc, DOM, POM, Competition, Temperature, pH, alkalinity, fish, crustacean

2.1. *Introduction*

Zinc and copper are essential heavy metals to aquatic organisms playing an important role in cellular metabolism acting as co-factors in a number of important enzymes (Gioda et al., 2007). The uptake of trace metals, according to (Rainbow, 1997), occurs from solution by one of two routes: passive facilitated diffusion (metal binds to a metal-binding protein in the membrane of the epithelial surface that facilitates its entrance) or active transport (following routes for the uptake of major metal ions; this uptake requires an energy pump in the epithelial cell membrane). The relative importance of different routes varies according to trace metals and between species.

However, these heavy metals are widely used in several anthropogenic activities, such as mineral, petrochemical and paint industries, and are frequently discharged at aquatic ecosystems, leading to toxic effects on organisms (Bianchini et al., 2005; Gauthier et al., 2006). Toxic effects include lethality or sublethal effects on reproduction and digestion, leading to fitness reductions of wild populations and/or indirect foodweb effects (Bianchini et al., 2005). The impact of the heavy metals is even greater in freshwaters than in marine waters (Bianchini et al., 2005).

Till this moment, performed standard toxicity tests are, generally, conducted at constant and favorable experimental conditions neglecting water physical and chemical variations that can alter toxic effects in organisms over several orders of magnitude (Santore et al., 2002). However, water quality is affected by the interactions of several organic and inorganic components, which are known to play an important role in metal toxicity by controlling its chemical speciation and bioavailability (Breault et al., 1996).

A proper assessment of the risk associated to the toxic effect in aquatic environment, by definition, is given by the free-ion portion activity of the trace metal which is the most bioavailable metal species (Kramer et al., 2004) capable of entering the organism and reaching an active site, provoking the toxic effect. Identification and comprehension of the role of these parameters is, hence, crucial to accurately predict toxicity in specific field situations.

Once the ultimate aim of ecotoxicological assays is to predict how the organisms respond to contaminants at natural conditions, physic and chemical composition of the water must be considered in ecotoxicological predictions (Chakoumakos et al., 1979).

In this paper we will discuss how the physic-chemical freshwater variables influence acute and chronic toxicity of zinc and copper metals. Possible implications of water chemistry, pH, temperature and organic content will be investigated for fishes and cladocerans (crustaceans), once differences between and within these groups to metal exposures have been demonstrated (e.g. Van Genderen et al., 2007). Possible differences between species will be related in order to incorporate in future developed biotic ligand models.

2.2. Relative importance of standard species

Ecotoxicological assays are usually conducted with standard species. Much of the available data is developed with fish species routinely produced in aquaculture facilities, such as trouts (Woodling et al., 2002). The biggest strength of using non indigenous species to determine metal contamination is that it produces data that can be used and understood by policy makers for making risk management decisions. On the other hand, the major problem of using non indigenous species to risk assessments is the lack of information on specific organisms and the current state of understanding on how an ecosystem functions (Stahl et al., 2001). In this context, the use of native species to determine the toxicity of natural waters has been questioned, especially when acclimated resident fish survive in the same water where exposure to native fish results in mortality. Several studies, such as the one of (Woodling et al., 2002), demonstrated that the use of standard species cannot always assure an appropriate water quality criteria about predictive effects of heavy metals in native lotic species.

However, acclimation to metals by fish is transient and will not persist when fish are removed from contaminated waters (Marr et al., 1996). In addition, the degree of resistance to metal exposure is variable, which makes comparison of experiments conducted in the same watershed over time problematic (Harper et

al., 2007). Given this, it is well understood why bioassays predicting toxicity are conducted with reference organisms that permit comparison between toxic effects on different waters.

Among freshwater animals, cladocerans are widely used in aquatic toxicology. In addition to their ecological significance the advantages are their length, short lifecycles, high fecundity and parthenogenic reproduction, ease of laboratory culturing, low space and water volume requirements and their sensitivity to toxicants. *Daphnia magna* Straus is probably the most commonly cladoceran used. However, its use has been criticized by several authors due to its limited geographical range and even within this range it is confined to small water bodies (Koivisto, 1995). Hence, its use to formulate water quality criteria (WQC) can only be justified if it can be demonstrated that *D. magna* has comparable sensitivity to that of more widespread, important members of zooplankton communities (intra and inter-species variations) (Bossuyt et al., 2005). An additional problem of the results obtained with daphnids is because they are frequently cultured in natural waters of unknown and fluctuable composition (Muyssen and Janssen, 2001).

2.3. The role of copper

Copper is an essential element for the survival of all aerobic organisms. Its redox nature is used in enzymatic processes, being a cofactor of several proteins involved in growth and development (FairweatherTait, 1997; Kamunde et al., 2002).

Copper is also a very potent toxicant when allowed to accumulate in excess of cellular needs (Kamunde et al., 2002). Its toxicity promotes physiological and behavioral stress due to reductions in food consumption or food assimilation (Lett et al., 1976) and also promotes increased metabolic costs associated with detoxification and ion homeostasis disruption (Marr et al., 1996).

Copper is a well-known disruptor of iono- and osmoregulatory activities in fishes. The primary mechanism of Cu toxicity, for fishes, results from the combined effects of plasma and whole body reduction in sodium (decreased influx and increased efflux of sodium) (Playle et al., 1993; Pyle et al., 2003) and of copper binding to histidine-, cysteine- and methionine-containing proteins which results in

their dysfunction (Kramer et al., 2004). Reduced Na^+ influx was associated to competitive and non-competitive binding of Cu ions to the Na^+/K^+-adenosine triphosphatase pump activity on the basolateral membrane of gill chloride cells, resulting in lower Na^+ uptake rates into the blood (Li et al., 1996; Pyle et al., 2003). Increased Na^+ efflux is thought to be associated with damages in gill epithelia (Pyle et al., 2003). The net loss of sodium seems to be dependent of both concentration and duration of copper exposure (Zahner et al., 2006).

In fish, at lethal copper exposure death usually occurs due to cardiac failure that is the final event of ionoregulatory and respiratory disturbances and physiological disorders including massive haemoconcentration and elevated arterial blood pressure respiratory disturbance (Erickson et al., 1996).

At Sublethal copper exposures, if fish survive to the initial shock or damage phase (that usually occurs in few days) then some physiological adjustments are done and fish can continue to exist (Hansen et al., 2002; McGeer et al., 2002). It appears to be a critical physiological threshold, beyond which an organism will be unable to recover and ultimately results in mortality (Zahner et al., 2006). Organisms respond to acclimation of copper by increasing biosynthesis processes (mitosis and protein synthesis) which help to repair the damage and correct the physiological disturbances. Increased metallothionein production to remove free metal ions, up-regulation of other pathways to counteract or compete with the deleterious effects of the metal, increased oxygen consumption, increased hemoglobin, plasma glucose and plasma cortisol and reduced mean swimming speed are mechanisms associated to the acclimation response (Dethloff et al., 1999; McGeer et al., 2000; McGeer et al., 2002; de Boeck et al., 2003; Handy, 2003). After that, if ionoregulatory mechanisms recover, the organism eventually should be able to re-establish hydromineral homeostasis and ultimately to survive. The internal physiology of the animal either returns to the pre-exposure condition or a new equilibrium is established leading to increased tolerance (McGeer et al., 2000; Taylor et al., 2000).

2.4. The role of zinc

Zinc has been less well studied than copper in both fish and crustaceans. It is an essential element for the survival of aquatic organisms (e.g. Caffrey and

Keating, 1997; Handy, 2003), playing a critical role in normal neuro-endocrine functions of vertebrates (Handy, 2003). At low concentrations, zinc is taken up actively from water mainly via the gill but also from dietary sources via the intestine being maintained in balance by fishes (Hogstrand et al., 1994; Hogstrand et al., 1996)) and cladocerans (Muyssen and Janssen, 2002).

However, zinc has been shown to be toxic to aquatic organisms when present at elevated concentrations (Santore et al., 2002). Its toxic effect varies according to the concentration and to the duration of this exposure (Woodling et al., 2002). Zinc interferes with the branchial calcium ion uptake both at lethal and sublethal concentrations because it probably shares the same apical entry mechanism of calcium (Hogstrand et al., 1994; Hogstrand et al., 1996).

The maintenance of Ca homeostasis in fish is crucial throughout life but is especially important in young fishes. Calcium is a critical element for growth (Pelgrom et al., 1997). Acute waterborne zinc toxicity, in fish, induces the net loss of calcium leading to decreased plasma calcium concentrations, and eventually to hypocalcemia (Santore et al., 2002). Zinc provokes inflammatory reaction manifested by physical damage to the branchial surface resulting in adverse respiratory response. When exposed to acute zinc concentrations, fish die as a result of tissue hypoxia secondary to impairment of gas exchange at the gill (Alsop et al., 1999).

Under sublethal zinc waterborne exposures fish can live due to an acclimation response. As in copper sublethal exposures, if fish survives to the initial damage phase to the gill epithelium, usually of 4 days, then some physiological adjustments are made which compensate for the altered environmental condition and confer increased tolerance to the metal (Hogstrand et al., 1994).

Such acclimation adjustments include alterations to gill barrier properties, reducing metal rate entry, increased metal storage and detoxification by linkage to metallothionein and increased resistance of metal-sensitivity processes to metal poisoning (Hogstrand et al., 1994; Alsop et al., 1999). Acclimation to this metal appears not to increase maintenance cost-of-life. Growth, whole-body Na^+ or Ca^{2+}

concentrations, metabolic rate, or fixed velocity swimming performance do not seem to be affected (de Schamphelaere and Janssen, 2004a).

For daphnids the same effect of zinc on calcium content was observed (Muyssen et al., 2006). The authors suggested that death, in daphnids, was, again, due to hypocalcaemia, once significant reductions in body burdens (reductions up to 37% of Ca body burden) at higher zinc concentrations were obtained. However, at sublethal reductions of Ca body contents, the movement and filtration rate were inhibited leading to a decreased food uptake and, hence, less energy available for the organisms to growth and reproduction. But after 14 days of exposure, general repair processes, such as restored normal calcium body content, normal feeding rates and available energy reserves, were conducted and daphnids acclimated to the sublethal zinc exposure. The researchers suggested that daphnids can correct Ca influx by restoration of functional transport sites and the system can be tuned to limit the influx of Zn by a persistent reduction in the affinities for both ions. As a result of these physiological alterations zinc concentration in body burdens can be reduced.

2.5. pH

The pH measures the basic or acid characteristic of water. It indicates the hydrogen ion concentration and is defined as the negative logarithm of the molar hydrogen ion concentration (-log $[H^+]$). Water is considered acidic when pH is below 7 and basic when pH is above 7 (Wurts and Durborrow, 1992).

The major effect of pH on metal toxicity seems not to be due to proton competition. Low values of cell membrane/gill surface stability constants (log K) at circumneutral pH 6.0 and 8.0 suggests that H^+ may not compete effectively for metal binding sites when present at equinormal concentrations to metal. The values suggest that at the gill microenvironment a high percentage of gill binding sites will be deprotonated and anionic. H^+ will only compete to the active sites of the organisms when H^+ concentrations are high enough to allow their competition by mass action (MacRae et al., 1999).

2.5.1. Copper

pH influences the speciation and bioavailability of copper, playing a key role in metal toxicity. Changes in metal speciation can alter the ability of metals to be adsorbed or alternatively to be absorbed by organisms. It is known that an increased proportion of Cu^{2+}, the most bioavailable copper species, exists under acidic conditions (pH <6). At circum-neutral pH values the most predominant copper species are hydroxide and carbonates, while Cu^{2+} concentrations are reduced. More basic conditions lead to the increase of carbonates (CO_3^{2-}) (Meyer, 1999) and copper hydroxides ($CuOH^+$ and $Cu(OH)_2^0$ species). Both carbonate and hydroxide species are dominant and the free ion concentration of copper (Cu^{2+}) is reduced (Chakoumakos et al., 1979; Stouthart et al., 1996). Given this assumption it is expected that metal toxicity decreases as the pH increases.

Inside the interval of 3.3 to 10 it was observed that the survival of *C. dubia* was not influenced by direct pH effects, except when pH was lowered to very low values (pH 3.0) (Kim et al., 2001). However, the addition of copper joint to increased pH from 7 to 10 resulted in lower LC_{50} values in *Ceriodaphnia dubia*. This increase in the LC_{50} was attributed to the precipitation and complexation of copper and the subsequent reduction of the free copper ions. Complementary, (Hyne et al., 2005) obtained reduced lethality in *C. dubia* when pH was increased from 5.5 to 7.5. These authors also observed that between pH 5.5 and 6.5 there was no significant difference in copper toxicity, suggesting sensitivity to the free cupric ion (Cu^{2+}), which was more dominant at acidic pH.

The same protective effect was observed to another cladoceran. *D. magna* was tested using a pH range between 5.98 and 7.92. The relation between pH and EC_{50} seemed to be curvilinear with saturating EC50 at higher H^+ concentrations (low pH values) (Long et al., 2004). Increasing EC_{50}'s for copper, with pH increase from 5.7 to 8.5, have been reported for *D. magna* (de Schamphelaere and Janssen, 2002). The researchers suggested that this effect could be, at least, partially related with speciation differences. If the relation between (H^+) and 48-h EC_{50} (Cu^{2+}) is considered as being linear, this would indicate the possibility of proton competition at the biotic ligand. However, data suggested a curvilinear

relation with saturating EC_{50} at higher pH. This situation led authors to suggest that probably copper hydroxides could partially produce toxic effects in organisms.

It has been proposed that at pH <6 proton binding to the gill can be significant because $[H^+]$ becomes high enough (10^{-5}) for protons to compete with Ca^{2+} and Cu^{2+} (Meyer, 1999). Therefore, at low alkalinity and hardness conditions, pH ~5 theoretically should result in higher LC_{50} values when compared to pH 6 as a result of competitive effect of H^+ protons for the binding sites of the organism. However, this protective effect of hydrogen, when present at certain high concentration, rapidly results in increased toxicity for heavy metals, once at low pH (pH = 4.0) it results in an additional toxic source, inhibiting severally sodium influx under high calcium concentrations. The result is a net loss of sodium content of daphnid (Glover and Wood, 2005).

Increased LC50 values in *D. magna were* observed with increase in pH between 6.5 and 8.5 (Van Sprang and Janssen, 2001) as a result of the decrease in toxic Cu^{2+} concentration, suggesting a stronger competition between Cu^{2+} and H^+ cations. At pH 8.5, the toxicity was drastically reduced. The acute protective effect of pH was also observed for fishes (e.g. Sciera et al., 2004). (Erickson et al., 1996) determined pH influence in fathead minnows ranged pH between 6.6 and 8.7 and observed significant 96-h cupric ion LC_{50}s and total copper LC_{50}s. On the total basis, copper LC_{50} increased with the pH increase. On the other hand, the cupric ion LC50, at low alkalinity, increased from pH 6.5 to pH 7.4, was approximately flat from pH 7.4 to pH 8.1, and decreased from pH 8.1 to pH 8.8. At high alkalinity conditions, cupric ion LC_{50}s showed some decrease from pH 7.2 to pH 8.0 and a steeper decrease from pH 8.0 to pH 8.6. The cupric ion LC_{50}s on the basis of cupric ion declined with increasing alkalinity, the rate of decline apparently increasing with increasing pH. Contrarily, LC_{50}s on the basis of cupric ion declined with increasing pH.

Studies on copper toxicity to early life stages of common carp (*Cyprinus carpo*), exposed to two different pH: 6.3 and 7.6 suggested that the pH increase resulted in protective effect to the organisms (Stouthart et al., 1996). At pH 7.6, Cu exposures did not affect the parameterized endpoints: egg mortality, heart rate, tail movements, and whole-body potassium and magnesium content. Although, a pH

reduction combined with the highest copper concentration, increased egg mortality and decreased heart rate and tail movements. It also led to premature hatching, increased larval mortality, and larval deformation. The authors suggested that the different toxic effect was due to increased Cu^{2+} concentration at lower water pH.

At a chronic level, it was observed that *D. magna* may vary significantly their NOECs (No Observed Effect Concentrations) and EC_{50}s due to pH differences (de Schamphelaere and Janssen, 2004b). Inside the pH range of 5.3-8.7 they observed that these two parameters increased linearly with the pH increase. This seems to contradict the observed protective effect that low pH (pH 5.0) may exert in acute copper toxicity reported above. At the other hand, it demonstrated the lower influence of competing hydrogen ions in reducing copper toxicity. However, Schwartz and Vigneault (2007) obtained opposite results with *Ceriodaphnia dubia* observed at copper chronic level. They observed a protective effect of the hydrogen ion. Hence, IC_{25} increased linearly with the concentration of hydrogen between the pH range of 5.6 and 8.0, when expressed as IC_{25} free Cu^{2+} activity. However, for low pH values (pH 5.6), the researchers also observed high mortality (40%) indicating a lower pH limit to the species.

2.5.2. Zinc

The pH is also an important parameter in zinc toxicity for both crustaceans and fishes. The acute zinc toxicity in *D. magna* shows a similar toxicity pattern to copper (Van Sprang and Janssen, 2001): inside the pH range of 6.5 and 8.5, the 24-h EC_{50} values were increased. The authors suggested changes in zinc speciation with pH variations, discussing that variations in 24-h EC_{50} values were due to free Zn^{2+} concentrations that decreased with increasing pH. They also discussed that at the tested lower pH values, the H^+ competitive effect was not sufficient to compete with the increased free Zn^{2+} cations for binding to the biologically active sites. However, the uptake rate of zinc in *D. magna*, under acute exposures, can decrease significantly with increasing acidity of the water from pH 7.0 to 5.0 as a result of the influence of higher H^+ concentrations (lower pH) on the biological binding sites (Yu and Wang, 2002). EC_{50} *D. magna* increase with the pH

increase from 6.0 to 8.0 (Heijerick et al., 2002). However, when EC_{50} was expressed as Zn^{2+} negligible impact of increased H^+ concentration was obtained, suggesting that H^+ would be a poor competitor for binding on the biotic ligand.

Zinc toxicity for *C. dubia* increases with pH values from 5.5 to 7.5 (Hyne et al., 2005). However, a pH variation from 5.5 to 6.5 did not result in significant toxicity variation possibly due, again, to the competitive effect of the hydrogen ion that removed the free zinc ions from the active sites.

pH influence in zinc toxicity for fishes was studied by (Santore et al., 2002). For fathead minnows LC_{50} values tend to decrease with increasing pH. They observed a U-shape pattern for rainbow trout with higher LC_{50} values at low (<6) and high pH (>8) and low LC_{50} values at intermediate pH. However, fathead minnow LC_{50} values tended to decrease with increasing Zinc solubility decreases with increasing pH to a minimum at approximately pH 8.5 where the solubility begins to increase due to the formation of soluble aqueous hydroxide species.

(Santore et al., 2002) reviewed the importance of water chemistry to aquatic organisms and observed that rainbow trout data suggested a U-shaped response of measured LC_{50} values to changes in pH. At low pH (<6) and also at high pH (>8) zinc LC_{50} values were elevated due to competition with protons at zinc binding sites in the first case and due to complexation reactions in the second case.

At a chronic level, pH increase from 6 to 8.5 resulted in a linear increase of predicted effect concentrations in *D. magna* (Heijerick et al., 2003). Later, (Heijerick et al., 2005) observed a linear increase in *D. magna* EC_{50}, when expressed as Zn^{2+}, with pH, when this variable was decreased from 8.0 to 5.5. However, the researchers suggested that pH effect would be better defined by a logarithmic function: a linear relationship with a steep slope at pH levels >7 and a linear relationship with a gentler slope at pH levels <7. These results seem to contradict the results obtained above. The authors suggested different mechanisms in acute and chronic exposure to zinc.

2.6. Natural Organic carbon

Natural organic matter (NOM) is a mixture of inorganic and organic compounds that show complexing properties (Paquin et al., 2000). Its chemical

composition may vary considerably between aquatic systems depending on the source of organic matter. This organic matter can be produced by phytoplankton (autochthonous NOM) or taken to the water from the surrounding catchment (allochthonous NOM). Autochthonous NOM is typically enriched in carbohydrate and nitrogen content while allochthonous NOM, highly colored, is enriched in aromatic humic and fulvic substances (Buffle, 1988). These compounds can bind protons and metal ions affecting metal bioavailability and transport (Dwane and Tipping, 1998). However, when present at elevated concentrations, the protective effect of natural organic matter is overlayed and it constitutes a source of additional acute stress and toxic reactions due to adverse effects in enzyme systems (Meems et al., 2004).

Dissolved organic matter (DOM), is the fraction of NOM constituted by organic matter that passes through a filter with a mesh of 0.45μm. DOC keeps metals in solution by binding them, preventing the solution's precipitating. It regulates the speciation of most bioactive metals, decreasing metal uptake and/or toxicity to freshwater organisms once it complexes the free metal ion, the most important available species (Erickson et al., 1996; de Schamphelaere et al., 2004; de Schamphelaere et al., 2005b). The formation of the metal-DOC complexes have been demonstrated that do not vary with the time from formation, i. e., the aged metal-DOC bonds do not better protect fish against accumulation or even if metal-DOC bonds do become stronger with time, the changes in metal-DOC binding strength are not biologically important (Hollis et al., 1996). This aspect is of major importance to determine metal-gill interactions for predictive exposures, indicating that the binding constants are robust.

It contains various organic ligands, such as humic, fulvic and hydrophilic acid, which exhibit different metal complexation behavior (de Schamphelaere et al., 2004). The relative abundance of these fractions vary considerably with water source traducing in different binding strength (or affinity) and complexation capacity (or site density) of aquatic DOM (de Schamphelaere et al., 2004; Bringolf et al., 2006). Within the organic ligands, the fulvic (FA) and humic (HA) acids are generally considered the most reactive fractions. Fulvic acids, tipically 40-80% of the total DOM, consist in greater number of proton-dissociating groups and fulvic

carboxyl groups have a more acidic nature. Consequently, the charge on FA is appreciably greater than that on HA. However, the greater molecular weight of HA, typically representing 4-30% of total DOC, means that they have a greater charge per molecule, typically 5 times of FA (Tipping, 1998).

In addition to the dissolved phase, natural surface waters contain suspended solids in the water column. Suspended solids are aggregated materials, including both biotic (phytoplankton and bacteria) and abiotic (inorganic and detrital organic matter) components. Although it is generally assumed that the dissolved fraction of a toxic substance in surface water is mainly responsible for toxicity to aquatic organisms, the suspended solids can play an important role in removing trace metals from the water column by chemical linkage or adsorption (Hart, 1982). As observed to DOM, the suspended solids can provoke additional toxic effects in cladocerans species. Cladocerans are non selective filter feeders and if ingested, suspended solids may act as a secondary stressor by reducing food assimilation or increasing body weight leading cladocerans to expend more energy to stay at the desired depth within the water column (Herbrandson et al., 2003).

2.6.1. Copper

In a general way, the presence of natural organic substances and suspended particles decrease the toxic effect of copper (e.g. Ma et al., 2002; Boeckman and Bidwell, 2006). Organic acids with much higher copper binding constants than that of the organism's active site can render a significant portion of the total copper non bioavailable. Hence, the protective effect of DOM must be primarily a function of its high metal binding capacity (e.g. Giesy et al., 1986).

Suspended solids can diminish metal bioavailability reducing metal effects on aquatic organisms (Boeckman and Bidwell, 2006; Taylor et al. 1998). In terms of kinetics, the reaction of copper with the suspension is faster than the reaction of copper with the filtrated water, i. e., dissolved organic matter, and the larger is the particle, the faster it settles (Ma et al., 2002). However, the adsorbed copper to suspended solids can be relatively toxic, being of almost half toxic that of dissolved copper (Erickson et al., 1996).

Dissolved organic matter reduces waterborne copper toxicity mainly by sequestering copper from biological uptake, reducing the chemical activity of the most toxic metal species in solution – the Cu^{2+} free bioavailable species (Kim et al., 2001; Kramer et al., 2004). DOM plays an additional physiological role on the active sites at fish gills, by increasing the Na^{+} transport capacity, sustaining higher Na^{+} uptake rates relative to Cu^{2+}. This results in sodium reduced net losses. This effect is more pronounced in hardwater than in softwater (Matsuo et al., 2004). Increasing DOC concentrations has been proved to decrease aquatic organisms' lethality. This protective effect was observed by (de Schamphelaere et al., 2004) in the 48-h LC_{50} for *D. magna* in a factor of more than 12 times. Additionally, (Ma et al., 1999) observed the same protective effect in the cladoceran *Ceriodaphnia dubia* lethality exposed to a commercial Humic Acid. They concluded that the toxicity of copper was directly correlated to the Cu^{2+}, existing a linear relationship between death and Cu^{2+} concentration. The same effect was observed with the experiments of (Villavicencio et al., 2005) that obtained higher copper LC_{50} values for *Daphnia obtusa* and *D. magna* at higher levels of natural DOC. Dissolved organic carbon concentration was also identified as a significantly copper reducer agent by (van Genderen, 2007) in natural waters to both *C. dubia* and *D. pulex* and accounted for approximately 75% of the variation observed in the datasets. However, the protective effect between DOC concentration and acute toxicity to *P. promelas* was also observed at DOC concentrations up to 4 mg/L.

Additionally, in fishes, the researchers also observed the same protective effect. (Erickson et al., 1996; Hammock et al., 2003) tested larval fathead minnow (*Pimephales promelas*) acute toxicity and HA concentrations up to 5mg/l, added as commercial humic acid, resulted in 90% complexation of copper-DOC leading to increased copper LC_{50}. However the authors did not achieve a particularly good correlation of toxicity to cupric ion lethality, relating it to some availability at the gills of organic complexes with different kinetics of dissociation and/or extent of dissociation. (Hollis et al., 1996) observed that DOC concentrations of ~5mg C/L could keep Cu off of rainbow trout (*Oncorhynchus mykiss*) gills. (McGeer et al., 2002) observed the protective effect of DOC in juvenile rainbow trout (*Oncorhynchus mykiss)*, which was associated to the reduced uptake of Cu into

the gills and liver. These authors also suggested that some inorganic and organic Cu complexes (as Cu-HA or CuOH) could be partially bioavailable because of the organisms' sensitivity to very low levels of Cu^{2+}. The Cu-HA complex, at the gills, could be dissociated and Cu^{2+} probably would be released from HA.

Evaluating the relative importance of DOM simply as a function of its concentration in water can possibly lead to inaccuracies because the NOM source can be also an important factor. Because of the complexity and heterogeneity of NOM, the ability to predict aqueous metal toxicity based upon modelled metal-NOM interactions may be inadequate (Ryan et al., 2004). Some authors suggested that characterizing the influence of dissolved organic matter only as a function of concentration can be problematic (e.g. Ryan et al., 2004). According to (Dwane and Tipping, 1998) different toxicities among water types may exist as a result of different natures of the DOM because of different FA ratios. This factor was observed by (de Schamphelaere et al., 2004) in acute *D. magna* tests. The authors observed 48-h EC_{50} for *D. magna* in a factor of 30 over the natural waters tested. (Richards et al., 2001) demonstrated that rainbow trout (*Oncorhynchus* mykiss) survival increased with the NOM concentration increase. They related it to increases in allochthonous properties of the water, i. e, increased concentrations of humic and fulvic content (most reactive species of DOM). (Ryan et al., 2004) also observed that the protective effect of dissolved organic carbon could only be correctly predicted if NOM source would be included. The authors tested 96-h static acute copper tests using fathead minnows (*Pimephales promelas*) under nine different water sources. They regretted LC_{50} against HA concentration (humic acid) and against NOM source observing significant differences between the different waters. Some sources of NOM had a significantly greater protective effect than others. The authors suggested that it would be required a parameter that further characterizes NOM quality, rather than just quantify it. The authors suggested that addicted to the measured humic fraction, the constituents in the fulvic and hydrophilic fractions of NOM may be important with respect to their ability to influence copper toxicity.

However, contradictory results were observed by (Kramer et al., 2004) that, using *D. magna*, found a linear correlation between DOC concentration and Cu^{2+}

concentration regardless of the surface of water tested. (Sciera et al., 2004) also concluded that DOC source, in a general way, did not significantly affect copper toxicity for fathead minnow (*Pimephales promelas*). However the researchers observed that, at pH 8, toxicity was also influenced by DOC source due to different copper speciation.

At a chronic level it was found that the DOM concentration is of great importance. However, its source seem to be of lower importance to *D. magna* toxic effect (de Schamphelaere and Janssen, 2004b). Although the three tested DOMs had different copper complexation capacities, they all probably protected against chronic copper toxicity to the same degree. The authors recognized that Cu-DOM complexes could be, partially, available for organisms. However they did not exclude that the exposure duration (acute versus chronic exposures), species tested, experimental setup (e. g. flow-through vs semistatic) or a combination of these conditions could influence the obtained results. (Schwartz and Vigneault, 2007) also found great correlations in *Ceriodaphnia dubia* chronic toxicity with commercial humic acid and natural organic matter concentrations, regardless to NOM source. The authors observed that DOM was the most protective effect against copper toxicity, when compared with other chemical variations (Mg, Ca, Na and pH) conducted in the experiments.

However, DOC concentration and, eventually, DOC source by themselves cannot be an accurate predictor of organic binding potential. DOC also interacts with water hardness. The binding site concentration changes with water hardness while binding strength of DOC remains constant. (Breault et al., 1996) observed that free Cu^{2+} was significantly lower in low hardness water, as compared with higher hardness (for samples adjusted to pH 6.25). However, the authors suggested that these differences could be reduced at ambient pH because of protonation and deprotonation of binding sites for low and high pH's, respectively.

2.6.2. Zinc

The natural organic matter role in modifying heavy metal toxicity is also extended to zinc toxicity. To better predict zinc toxic effects, it is crucial that speciation of zinc (i.e., the activity of free Zn^{2+}) can be correctly predicted in the

presence of natural DOC because the free Zn^{2+} ion is considered the most bioavailable metal species (de Schamphelaere et al., 2005a).

It was demonstrated that suspended contaminated particles (such as algae, sand, peat and clay) can exert additional acute toxic effects that cannot be explained by the dissolved concentration of the zinc on their physical presence (Weltens et al., 2000). The authors justified it as possible bioavailability of the metal within the body of *D. magna* that are continuously filtering the surrounding water.

In terms of dissolved organic matter, it was observed that lower DOC concentrations resulted in lower concentration of complexed metal with DOC (Cheng et al., 2005). (Hammock et al., 2003) observed that the addition of HA reduced zinc uptake in Chinook salmoon (*Oncorhynchus tshawytscha*) eggs. (Bringolf et al., 2006) tested 96-h acute zinc toxicity in fathead minnow larvae and concluded that Zn toxicity was influenced by DOM concentration from a variety of surface waters on a similar way. A threshold of ~11 mg DOC/L was needed to exert protective effect against Zn toxicity: above this value, 96-h Zn LC_{50} was linearly related to DOC concentration. These achieves seem to confirm the results obtained by (Hyne et al., 2005) which observed that the addition of 10 mg/L DOC only resulted in a very small reduction in the toxicity of zinc to *C. dubia*. Complementary, (Santore et al., 2002) did not observe zinc LC_{50} variations with DOC increase in the reviewed data for rainbow trout, which confirm the results presented above because the reported DOC concentrations were low.

(Cheng and Allen, 2006) suggested that the origin of natural organic matter would not interfere with the protection effect against zinc. One set of binding constants and ligand concentrations could be used to represent Zn-NOM complexation for NOM from different surface water. They related it to similar main mechanisms of metal binding, the metal complexation with carboxylic groups and phenolic groups.

At a zinc chronic level in *D. magna,* (Heijerick et al., 2003) showed that DOC concentration appears to be the most important toxicity modifying factor for Zn toxicity. (de Schamphelaere et al., 2005a) found similar conclusions in both chronic and acute response for crustaceans and fishes. In the tested water

samples they observed that DOC was the most protective effect against zinc at acute and chronic levels for *Daphnia magna* (48-h immobility tests and 21-d reproduction tests) and *Oncorhynchus mykiss* (30d mortality and growth) and they did not obtain significant differences in zinc toxicity due to water type. They eliminated water source effect by choosing a certain percentage of active fulvic acid. Additionally, they also observed that DOC was more protective for chronic than for acute exposures because at lower Zn concentrations, more Zn was complexed to DOC.

The protective effect of DOM in zinc toxicity is lower when compared to its effect in copper toxicity. It is needed more DOM concentration to exert the same acute protective effect on zinc (Bringolf et al., 2006). The authors suggested that it occurs because Cu is much more acutely toxic to the generality of aquatic organisms than Zn (copper LC_{50} is lower than zinc LC50 for both fishes and crustaceans) and Cu binds relatively strongly to DOM (Cu binding characteristics of humic acid and/or fulvic acid). The result is that less DOM is needed to complex Cu than is needed to complex Zn at concentrations near the $ZnLC_{50}$. These results were in accordance to the results obtained by (Hyne et al., 2005) that just observed slight significant decreases in zinc toxicity for *C. dubia* when DOC was present at 10 mg/L, while DOC concentrations of 5mg/L are needed to reduce copper toxicity.

2.7. *Chemical composition of the medium*

2.7.1. Copper toxicity

Several authors have demonstrated that copper toxicity decreases with water hardness increase. It is believed that hardness is a key element on toxicity tests and is defined as a measure of the quantity of divalent ions such as calcium, magnesium and/or iron in water. Calcium and magnesium are usually the most common sources of water hardness. The hardness of a water sample is reported in milligrams per litre as calcium carbonate (mg/L $CaCO_3$) (Wurts and Durborrow, 1992). Hardness influence was recognized to be an important parameter and it was incorporated by the regulatory policies (USEPA, 1993).

At acute level, the generality of the tests observed a protective effect of major ions against toxic effect (e.g. Villavicencio et al., 2005). Hardness is believed to influence acute copper toxicity by two modes: reducing gill uptake of copper through competition for biotic binding sites with calcium (Pyle et al., 2003; de Schamphelaere and Janssen, 2004b), and stimulating the activity of the cell surface Ca pumps reducing ion loss (Rainbow, 1997). In terms of competition reactions at the gill, Ca^{2+} is a poor competitor of Cu^{2+} for binding sites. Although Ca^{2+} binding to biological ligands on the surface of organisms is weaker than Cu^{2+}, its overall effect may still be significant because calcium is present at significantly higher concentrations than heavy metals in natural environments (Kim et al., 2001).

The acute toxicity of *D. magna* among different clones has been inversely related to water hardness (Barata et al., 1998). Although copper speciation predictions at moderate-hard and hard water were dominated by the $CuCO_3$ with minor proportions of $CuOH^+$ and Cu^{2+}, the free copper ion (Cu^{2+}) accounted for the biggest environmental variability in the toxicity of copper. (Long et al., 2004) also observed that copper toxicity in *D. magna* was inversely related with hardness (below than 50 mg/L as $CaCO_3$), following a linear trend. This inverse effect of hardness with copper was also observed in *Ceriodaphnia dubia* 24-h LC50 (Kim et al., 2001). However, at very high hardness (ranging from approximately 300 to 1200 mg/l as $CaCO_3$) reconstituted waters in copper LC50 of *Ceriodaphnia dubia* generally did not change as a function of hardness or as a function of calcium. However, both magnesium and sodium concentrations were good linear predictors of copper toxicity. The authors suggested that *C. dubia* is not very sensitive to hardness-dependent changes in copper toxicity compared to other cladocerans (Gensemer et al., 2002).

The same protective effect of increased hardness was observed to fish by (Erickson et al., 1996). Increased hardness concentration resulted in protective effect against copper toxicity in rainbow trout (*Pimephales promelas*), but the incremental effect decreased at higher hardness and LC_{50} reached an asymptote. The researchers suggested that toxicity could not be simply explained by the occupation of critical proportions of the gill binding sites occupied by copper.

However, when copper was expressed in terms of cupric ion the results were inconsistent with the hardness addition. They suggested that a variety of copper species could be contributing to toxicity. Sodium concentration was also identified to be correlated with the toxic effect. The researchers additionally tested different Ca: Mg ratios but did not observe significant differences in LC_{50} values. However, data were near the flattened portion of the hardness concentration *vs* copper LC_{50} curve, which was considered uncertain about the relative effects of calcium and magnesium in copper toxicity protection.

(Van Genderen et al., 2007) tested the *C. dubia* and *D. pulex* (invertebrates) and the fathead minnow *P. promelas* (fish) in natural waters ranging in terms of total hardness and alkalinity from 65 to 1200 mg/L as $CaCO_3$, and 16 to 230 mg/L as $CaCO_3$, respectively. The obtained data suggested no relationship between total hardness and alkalinity as well as no significant relationship between Cu LC_{50} values and Ca or Mg. On the other hand, sodium concentrations were significantly correlated with Cu LC_{50} values for the invertebrates (*C. dubia* and *D. pulex*) but the same protective effect was not observed to *P. promelas*.

The hardness studies do not explain how the protective effect of hardness occurs due to not specify the relative importance of calcium and magnesium concentrations on the protection against the toxic effect. Consequently, some research was conducted in order to determine if magnesium and calcium exerted the same protective effect to the organisms. (Naddy et al., 2002) suggested that very hard waters with manipulating Ca: Mg ratios could possibly produce distinct protective effects for organisms. They observed that different Ca: Mg ratios at hard water (at constant alkalinity 120mg/L as $CaCO_3$) had different protective effect against copper acute toxicity for five aquatic species. Copper toxicity increased to copper at lower Ca: Mg ratios for rainbow trout but increased at higher Ca: Mg ratios for *D. magna*. Fathead minnows (<24h) were more sensitive to copper in 1:1 Ca: Mg waters compared to 3:1 Ca: Mg waters. And, finally, copper under different Ca: Mg ratios for *Gammarus* sp., *C. dubia*, and 28-d-old fathead minnows. The results of (Van Genderen et al., 2007) showed that while increasing hardness from 200 to 1000 mg/L as Ca or Mg (between 0.1-6.0) had a protective effect from Cu

toxicity to *C. dubia* and *D. pulex* for *P. Promelas* Ca had a protective effect as Mg exerted no significant impact from acute Cu toxicity.

Additionally, (Perschbacher and Wurts, 1999) tested the individual effect of calcium or magnesium hardness on the acute toxicity of 48-h LC_{50} copper to juvenile channel catfish (*Ictalurus punctatus*) in low alkalinity environments. They observed that increased calcium hardness from 10 to 400 mg/L CaCO3 severally decreased fish lethality (from 90 to 5%). They also observed that hardness increases (as Ca^{2+} addition) from 200 to 400 mg/l were not significantly different. Magnesium-based hardness between 20 and 400 mg/l CaCO3 resulted in 100% mortality. Survivals were 48 and 100% in 20 and 400 mg/l calcium hardness treatments. The authors suggested that this could be associated with a calcium-specific mechanism with respect to acute copper toxicity in juvenile channel catfish. This study showed that high ration of the concentrations of calcium to copper ions would minimize the toxic effects of copper (by reducing or preventing competitive inhibition). At the other hand, magnesium hardness addition provided no protection from copper toxicity.

Varying each major cation individually, (de Schamphelaere and Janssen, 2002) obtained positive linear relations between EC50 (Cu^{2+}) and activities of Ca^{2+}, Mg^{2+}, and Na^{+}. Only potassium (up to 2mM) did not significantly affect acute copper toxicity for *D. magna*. Additionally, neither sulphate nor chloride ions had any effect on acute copper toxicity.

At a chronic copper level in *D. magna* water hardness (ranged from 25-500 mg/L as CaCO3) did not exert significant effects in copper toxicity (de Schamphelaere and Janssen, 2004b). The authors explained the lower importance of this competitive interaction to the increase in exposure time and to the decrease in metal concentrations required to elicit the sublethal effect. In contrast to acute copper expositions, no significant Ca^{2+}, Mg^{2+} or combined competition effect was observed in the chronic copper exposures. This lower role attributed to hardness at a chronic level confirm the previous results obtained by (Winner, 1985) that even obtained a little increase in copper toxicity with water hardness increase. They justified these results by possible competitive linkage with copper to the organic matter. (Schwartz and Vigneault, 2007), recently, also

tested the influence of individual effects of major ions: Ca, Na and Mg in protecting against copper chronic toxicity in *Ceriodaphnia dubia*, expressed as IC_{25}. They, again, found no significant protective effect of Ca and Na to copper chronic toxicity, and just small protective effect of Mg.

Sub-chronic experiments conducted with the rainbow trout (*Oncorhynchus mykiss*) concluded that the relationship between growth response and hardness (derived from several studies) appeared to be stronger than the relationship between hardness and acute copper response (Hansen et al., 2002).

2.7.2. Zinc toxicity

Zinc toxicity can also be reduced several folds as a consequence of the presence of competing hardness ions present in the water. Tests with *D. magna* in single-metal solutions of Zn under conditions of varying water hardness from soft to hard water for periods ranging from 12-96h showed that Zn acute toxicity was inversely related to water hardness due to hardness ions (Mg^{2+} and Ca^{2+}) increase, which competed with the free ion metal at the cell membrane surface (Barata et al., 1998). However, in this experiment, the effect of hardness could not be isolated because alkalinity (increased by the same proportion as hardness increased) and pH (from 7.2 to 8.07) also varied with the water hardness. The same protective effect of Ca^{2+} was obtained by (Yu and Wang, 2002) which concluded that zinc influx rates of *Daphnia magna* decreased as a function of the amount of Ca^{2+} present in solution. The same researchers observed that the uptake rates of the metal were not significantly affected by the concentration of SO_4^{2-}. On the other hand, any change in Ca^{2+} or SO_4^{2-} as well as pH did not apparently affect the distribution of zinc in the daphnids. (Heijerick et al., 2002) tested the individual effect of different major cations (Ca^{2+}, Mg^{2+}, Na^{+} and K^{+}) on the acute toxicity of zinc in synthetic waters. A significant decrease in zinc toxicity to the waterflea *D. magna* was observed due to increased concentrations of Ca^{2+}, Mg^{2+} and Na^{+}. The 48-h EC_{50} results suggest that Ca^{2+}, Mg^{2+} and Na^{+} can compete with Zn^{2+} for binding on the biotic ligand and will decrease Zn toxicity when present in high concentrations. However, at higher Ca^{2+} and Mg^{2+} activities (very hard water), the 48-h EC_{50} seems remaining constant. The authors proposed that high activities of these two ions would result in adverse effects on the daphnid

physiology and supported it by decreased *D. magna* reproduction rates obtained in unpublished data. *C. dubia* is also protected two- to threefold against zinc toxicity when water hardness is increased (Hyne et al., 2005).

(Santore et al., 2002) reviewed data of rainbow trout, fathead minnow and *D. magna* from several authors and concluded that while increasing either calcium or magnesium in the water may provide a competitive benefit by reducing the degree of interaction of zinc at the gill, an increase in calcium may have the additional benefit of facilitating calcium uptake when this saturable process is operating at a less than maximum rate. For rainbow trout they observed a notable trend of increasing zinc LC_{50} value with increasing hardness due to competitive effects between hardness cations and zinc on the biotic ligand. However, the authors observed that few of the analyzed rainbow trout studies reported measurements of calcium or magnesium. They also concluded that Zinc LC_{50} values did not appear to be correlated with other chemical parameters (alkalinity and sulfate) but co-variation between chemical parameters in these data could hide the effects of these parameters. The results of *D. magna* indicated that zinc LC_{50} were lower comparing to the observed for fishes. As in fishes, measured zinc LC_{50} values were correlated with hardness, while the effects of other parameters were weaker and likely confounded by co-varying hardness concentrations.

The influence of major cations was also demonstrated at a chronic level. The isolated effects of the major ions Ca, Mg and Na on zinc toxicity were tested in juvenile rainbow trout (*Oncorhynchus mykiss*) 35-42d old, using 30-d assays in which survival and growth were monitored. Ca^{2+} observed to be the most important accounting to the toxicity reduction, followed by Mg^{2+}, Na^{+} and H^{+} (added as pH variations) (de Schamphelaere and Janssen, 2004a).

The cladoceran *D. magna* was also tested at a chronic level by (Heijerick et al., 2003), which tested hardness of natural waters over a range test 35-445 mg/L as $CaCO_3$. An inverse U-shaped relationship was observed for the hardness toxicity. Zn toxicity seemed to be influenced by both changes in competition between Zn^{2+} and Ca^{2+}/Mg^{2+} ions and water hardness on the general health of the daphnids. The reduction in Zn EC_{10} at high hardness (>300mg/L as CaCO3) was

attributed to Ca or Mg complexation to binding sites on humic acid, leading to higher bioavailable Zn fractions and, hence, resulting in lower 21d-EC_{10}.

(Heijerick et al., 2005) observed protective effect again for *D. magna* with increasing concentrations of calcium and magnesium and a smaller protective effect of sodium. However, higher Na^+ concentrations were not considered by the authors because previous results had shown that high Na^+ content could induce additional stress, making organisms more sensitive to zinc.

Comparable effect of the physic-chemical elements affecting Zn and Cu toxicity have been suggested (Heijerick et al., 2002). The main difference between these two metals seem to be the large difference between the stability constants for the two metals that would reflect the higher toxicity and binding affinity of copper compared with zinc. At a chronic level, however, major cations (such as Ca^{2+}. Mg^{2+} and Na^+) seem not to influence copper toxicity, while a protective effect is observed for zinc.

2.8. The effect of Alkalinity

2.8.1. Copper

Alkalinity is the quantity of base present in water. Common bases found in water include carbonates, bicarbonates, hydroxides, phosphates and berates. Carbonates and bicarbonates are the most common and most important components of alkalinity. Total alkalinity is usually expressed as milligrams per litre calcium carbonate (mg/L $CaCO_3$) (Wurts and Durborrow, 1992). As pH, it influences metal speciation reducing its toxicity by decreasing the free metal ion, the most bioavailable species (Erickson et al., 1996). However few studies about its direct influence on metal toxicity are available because alkalinity and hardness are usually strongly autocorrelated in most natural and reconstituted waters (Gensemer et al., 2002) and its protective effect in reducing metal toxicity is confounded with hardness effects. However, these water parameters are not always autocorrelated and some studies demonstrated that isolated effects of increased alkalinity can have implications in copper toxicity.

Combined low hardness and alkalinity concentrations (e.g., ≤1mg/L as $CaCO_3$), results in negligible metal complexation with HCO_3^- and CO_3^{2-} and competition by Ca^{2+} for metal-binding sites will be minimal because of the large percentage of unoccupied binding sites on the gill (Meyer, 1999). But if hardness and alkalinity are extremely high (e.g. >1000 mgL-1 as $CaCO_3$), a large percentage of the metal in solution is complexed with HCO_3^- or CO_3^{2-}, and the Metal-binding sites on the gill are almost completely occupied by Ca^{2+} leading to lower toxic effects on the organisms.

Increased alkalinity (from low to high values) of synthetic freshwaters, added as sodium carbonate, can result in decreased toxicity of copper to *C. dubia* possibly by the formation of copper-carbonate complexes (Hyne et al., 2005). However, with the alkalinity modification, the pH of the treatment solutions was changed.

(Chakoumakos et al., 1979) presented one work that tested its isolated effect. They tested the isolated influence of alkalinity and hardness in the acute toxicity of copper (96-h LC_{50}) in cutthroat trout *(Salmo clarhi*) and observed effective copper LC_{50} increase with alkalinity increases. The protective effect of alkalinity was attributed, in part, to copper speciation. The same effect in copper toxicity reduction was observed by (Gensemer et al., 2002) witch observed significant increases in copper LC_{50} due to free metal ion complexation by carbonates and bicarbonates in solution.

Significant effects of alkalinity in *P. promelas* (fathead minnow) LC_{50} values for both total and free copper ion have been observed (Erickson et al., 1996). However, while increased alkalinities resulted in linear increases of LC_{50} total copper, this relation was not observed when alkalinity was regretted against free copper ion. They justified the unsuccessful regression by limited amount of data combined with experimental variability, errors in stability constants and uncertainties in copper speciation. The authors referred also that copper speciation could not be changed independently of other water chemical characteristics, such as pH, that also influence organism's toxic response.

2.8.2. Zinc

Zinc is weakly hydrolyzed at pH 7-8 and possesses relatively weak binding affinities with inorganic ligands. As a result, the speciation of zinc is dominated by the free hydrated metal ion (Zn^{2+}) (Smith, 1995 in Barata 1998) and only little variations in zinc toxicity are expected as a result of increased alkalinity and, consequently, lower concentrations of free zinc ion.

At our knowledge, few studies tested the individual effect of alkalinity in zinc toxicity. (Santore et al., 2002) reviewed data for rainbow trout did not observe significant zinc LC50 correlations with alkalinity but (Hyne et al., 2005), oppositely, observed significant reductions in zinc toxicity when alkalinity of synthetic water was ranged from low to high alkalinity in *C. dubia*.

2.9. Temperature

Most aquatic organisms are ectotherms, which makes temperature an important environmental factor controlling physiological processes, which may have great effects on the exposure of organisms to toxicants (Boeckman and Bidwell, 2006). Although being identified from several decades, this environmental variable has been less studied when compared to other water quality variables reported above.

In nearly all experiments thermal stress increased the adverse effects of substances (Heugens et al., 2002). As temperature influences all kinds of physiological processes, an interaction between temperature and toxicants can be expected when a chemical acts on these processes. It may affect uptake, elimination, and detoxication rates because of changes in metabolic, locomotory, and feeding activity of organisms (Boeckman and Bidwell, 2006).

Generally, organisms have associated temperature optima and chemical stressors exposure on either side of this range can influence their response (Boeckman and Bidwell, 2006). Low and high temperatures have been reported to increase toxicity. Temperature variations, alone, may alter the ingestions rates for cladocerans (Yurista, 1999; Loiterton et al., 2004). It has been suggested that high temperature values (30º C) are not enough to cause death but are sufficient to

result in starvation of the individuals due to accelerated respiration and low feeding which significantly reduces growth and fecundity (Lye Koh et al., 1997). This could result in low daphnids' viability in high temperature habitats that typically occur in summer or after discharges of some industries than can raise the ambient temperature by 8 or 9º C.

The population growth of *M. macrocopa* is optimal at the temperature interval between 20 and 25ºC (Benider et al., 2002). Above this interval, an increase in temperature resulted in shorter life span so that females allocate more energy for reproduction. Below the interval, fecundity is reduced and hence survival improved. (Seidl et al., 2005) did not observe significant influence in *D. magna* growth when temperature increased from 20º to 30ºC. However, at high temperatures daphnids had a shortened reproductive cycle in comparison to 20ºC acclimated ones.

Heavy metals (zinc and copper) increase their toxic effect in *Moina macrocopa* with the increase of temperature from 22º to 27º C (Nandini et al., 2007). These authors observed a reduction in survival and reproduction (resulting in reductions in average lifespan) in the cladocerans exposed to copper and zinc at higher temperatures. They also observed that copper was more adverse to *M. macrocopa* than zinc as was reported above. However, contradictory results were obtained with *D. pulex* for the effect temperature (Boeckman and Bidwell, 2006). In their experiments, temperature variation from 10 to 30ºC had little influence on *D. pulex* response to copper exposition, while it did for *Diaptomus clavipes* (a calanoid copepod). Raising the bioassay temperature to 30ºC resulted in a significant increase in the sensitivity of *D. clavipes* to copper. The apparent lack of temperature influence on *D. pulex* was explained by the authors as a possible statistical artifact since, at least for the total copper LC_{50} values, there was a decrease between 20 and 30ºC, indicating increased sensitivity to the metal at the higher temperature.

Increasing toxicity of copper sulphate to channel catfish with decreasing temperatures from 27-21 ºC has been reported (Perschbacher, 2005). The 72-h was significantly lower in the 27º C treatment compared to the 21, 23 and 25º C. The authors suggested that, at lower temperatures, the resisting mechanisms for

copper could be reduced. They speculated that this inverse relationship could be due to decreased enzyme production, levels and rates of activity; decreased membrane transport from the body; and decreased mucus production to inactivate copper. On the other hand, the authors observed that survival time decreased with increasing temperatures.

2.10. Acclimation of organisms

The importance of the acclimation period is recognized by several authors that can increase metal tolerance in toxicity tests performed with both daphnids (Bossuyt and Janssen, 2003; Heijerick et al., 2003) and fishes (Harper et al., 2007). In *D. magna* (Muyssen et al., 2002) showed that the acclimation to metals could also lead to different reproductive strategies (fewer offsprings with more maternal energy reserves vs more offsprings with less maternal energy reserves) and different body lengths. This aspect can be of major importance when testing sublethal metal concentrations However, (Muyssen and Janssen, 2005) results with *D. magna* did not show significant changes in the acute zinc tolerance with varying water culture characteristics.

2.11. Summary and Future directions

Zinc and copper are two common contaminants of the water bodies. However, different water characteristics can vary the toxic effects of these two metals to the biota. In this review, we reported several studies about their impact in copper and zinc toxicity for both fish and cladocerans. pH and alkalinity can influence copper and zinc toxicity because of their role in metal speciation. In fact, alkalinity and pH increases can result in lower concentrations of the most toxic species, the free metal ion. Major cations (such as calcium, magnesium and calcium) and hydrogen, when presented at high concentration can compete with the heavy metals, excluding them from the active sites of the organisms. However, at chronic copper expositions, the protective effect of the major ions is not so clear and more research is necessary. The organic matter content is also an important parameter when evaluating metal toxicity. Indeed, not only the dissolved fraction should be considered but also the particulate fraction, once few studies

demonstrated that the particulate matter can be a source or a sink of metal toxicity. However, more research is necessary to reliably determine its influence. Temperature is also a limiting factor of the organisms. Organisms have an optimal temperature range and outside certain limits the toxic action of the metals is increased.

2.12. References

Alsop, D.H., McGeer, J.C., McDonald, D.G., Wood, C.M., 1999. Costs of chronic waterborne zinc exposure and the consequences of zinc acclimation on the gill/zinc interactions of rainbow trout in hard and soft water. Environ. Toxicol. Chem. 18, 1014-1025.

Barata, C., Baird, D.J., Markich, S.J., 1998. Influence of genetic and environmental factors on the tolerance of *Daphnia magna* Straus to essential and non-essential metals. Aquat. Toxicol. 42, 115-137.

Benider, A., Tifnouti, A., Pourriot, R., 2002. Growth of *Moina macrocopa* (Straus 1820) (Crustacea, Cladocera): influence of trophic conditions, population density and temperature. Hydrobiologia 468, 1-11.

Bianchini, A., Martins, S.E.G., Barcarolli, I.F., 2005. Mechanism of acute copper toxicity in euryhaline crustaceans: implications for the Biotic Ligand Model. Animals and Environment 1275, 189-194.

Boeckman, C., Bidwell, J., 2006. The Effects of Temperature, Suspended Solids, and Organic Carbon on Copper Toxicity to Two Aquatic Invertebrates. Water Air Soil Poll. 171, 185-202.

Bossuyt, B.T.A., Janssen, C.R., 2003. Acclimation of Daphnia magna to environmentally realistic copper concentrations. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 136, 253-264.

Bossuyt, B.T.A., Muyssen, B.T.A., Janssen, C.R., 2005. Relevance of generic and site-specific species sensitivity distributions in the current risk assessment procedures for copper and zinc. Environ. Toxicol. Chem. 24, 470–478.

Breault, R.F., Colman, J.A., Aiken, G.R., McKnight, D., 1996. Copper Speciation and Binding by Organic Matter in Copper-Contaminated Streamwater. Environ. Sci. Technol. 30, 3477-3486.

Bringolf, R., Morris, B., Boese, C., Santore, R., Allen, H., Meyer, J., 2006. Influence of Dissolved Organic Matter on Acute Toxicity of Zinc to Larval Fathead Minnows (*Pimephales promelas*). Arch. Environ. Con. Tox. 51, 438-444.

Buffle, J., 1988. Complexation reactions in aquatic systems. An analytical approach. John Wiley and Sons,New York, NY, United States.

Caffrey, P.B., Keating, K.I., 1997. Results of Zinc deprivation in daphnid culture. Environ. Toxicol. Chem. 16, 572-575.

Chakoumakos, C., Russo, R.C., Thurston, R.V., 1979. Toxicity of copper to cutthroat trout (*Salmo clarki*) under different conditions of alkalinity, pH and hardness. Environ. Sci. Technol. 13, 213-219.

Cheng, T., Allen, H.E., 2006. Comparison of zinc complexation properties of dissolved natural organic matter from different surface waters. J. Environ. Manage. 80, 222-229.

Cheng, T., Schamphelaere, K.D., Lofts, S., Janssen, C., Allen, H.E., 2005. Measurement and computation of zinc binding to natural dissolved organic matter in European surface waters. Anal. Chim. Acta 542, 230-239.

de Boeck, B., de Wachter, B., Vlaeminck, A., Blust, R., 2003. Effect of cortisol treatment and/or sublethal copper exposure on copper uptake and heat shock protein levels in common carp, *Cyprinus carpio*. Environ. Toxicol. Chem. 22, 1122-1126.

de Schamphelaere, K.A.C., Janssen, C.R., 2002. A Biotic Ligand Model Predicting Acute Copper Toxicity for *Daphnia magna*: The Effects of Calcium, Magnesium, Sodium, Potassium, and pH. Environ. Sci. Technol. 36, 48-54.

de Schamphelaere, K.A.C., Janssen, C.R., 2004a. Bioavailability and Chronic Toxicity of Zinc to Juvenile Rainbow Trout (*Oncorhynchus mykiss*):

Comparison with Other Fish Species and Development of a Biotic Ligand Model. Environ. Sci. Technol. 38, 6201-6209.

de Schamphelaere, K.A.C., Janssen, C.R., 2004b. Effects of dissolved organic carbon concentration and source, pH, and water hardness on chronic toxicity of copper to *Daphnia magna*. Environ. Toxicol. Chem. 23, 1115-1122.

de Schamphelaere, K.A.C., Lofts, S., Janssen, C.R., 2005a. Bioavailability models for predicting acute and chronic toxicity of zinc to algae, daphnids, and fish in natural surface waters. Environ. Toxicol. Chem. 24, 1190-1197.

de Schamphelaere, K.A.C., Unamuno, V.I.R., Tack, F.M.G., Vanderdeelen, J., Janssen, C.R., 2005b. Reverse osmosis sampling does not affect the protective effect of dissolved organic matter on copper and zinc toxicity to freshwater organisms. Chemosphere 58, 653-658.

de Schamphelaere, K.A.C., Vasconcelos, F.M., Tack, F.M.G., Allen, H.E., Janssen, C.R., 2004. Effect of dissolved organic matter source on acute copper toxicity to *Daphnia magna*. Environ. Toxicol. Chem. 23, 1248-1255.

Dethloff, G.M., Schlenk, D., Khan, S., Bailey, H.C., 1999. The Effects of Copper on Blood and Biochemical Parameters of Rainbow Trout (*Oncorhynchus mykiss*). Arch. Environ. Con. Tox. 36, 415-423.

Dwane, G.C., Tipping, E., 1998. Testing a humic speciation model by titration of copper-amended natural waters. Environ. Int. 24, 609-616.

Erickson, R.J., Benoit, D.A., Mattson, V.R., Nelson Jr, H.P., Leonard, E.N., 1996. The effects of water chemistry on the toxicity of copper to fathead minnows. Environ. Toxicol. Chem. 15, 181-193.

FairweatherTait, S.J., 1997. Bioavailability of copper. Eur. J. Clin. Nutr. 51, S24-S26.

Gauthier, C., Couture, P., Pyle, G.G., 2006. Metal effects on fathead minnows (*Pimephales promelas*) under field and laboratory conditions. Ecotox. Environ. Safe. 63, 353-364.

Gensemer, R.W., Naddy, R.B., Stubblefield, W.A., Hockett, J.R., Santore, R., Paquin, P., 2002. Evaluating the role of ion composition on the toxicity of copper to *Ceriodaphnia dubia* in very hard waters. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 133, 87-97.

Giesy, J.P., Alberts, J.J., Evans, D.W., 1986. Conditional statbility constants and binding capacities for copper (II) by dissolved organc carbon isolated from surface waters of the Southeastern United States. Environ. Toxicol. Chem. 5, 139-154.

Gioda, C.R., da Rocha, J.B.T., Morsch, V.M., Schetinger, M.R.C., Lissner, L.A., Pretto, A., Neto, J.R., Loro, V.L., 2007. Exposure to sublethal concentrations of Zn(II) and Cu(II) changes biochemical parameters in *Leporinus obtusidens*. Chemosphere 69, 170-175.

Glover, C.N., Wood, C.M., 2005. Physiological characterisation of a pH- and calcium-dependent sodium uptake mechanism in the freshwater crustacean, *Daphnia magna*. The Journal of Experimental Biology 208, 951-959.

Hammock, D., Huang, C.C., Mort, G., Swinehart, J.H., 2003. The Effect of Humic Acid on the Uptake of Mercury(II), Cadmium(II), and Zinc(II) by Chinook Salmon (*Oncorhynchus tshawytscha*) Eggs. Arch. Environ. Con. Tox. 44, 0083-0088.

Handy, R.D., 2003. Chronic effects of copper exposure versus endocrine toxicity: two sides of the same toxicological process? Comp. Biochem. Phys. A 135, 25-38.

Hansen, J.A., Lipton, J., Welsh, P.G., Morris, J., Cacela, D., Suedkamp, M.J., 2002. Relationship between exposure duration, tissue residues, growth, and mortality in rainbow trout (*Oncorhynchus mykiss*) juveniles sub-chronically exposed to copper. Aquat. Toxicol. 58, 175-188.

Harper, D., Farag, A., Brumbaugh, W., 2007. Effects of Acclimation on the Toxicity of Stream Water Contaminated with Zinc and Cadmium to Juvenile Cutthroat Trout. Arch. Environ. Con. Tox.

Hart, B., 1982. Uptake of trace metals by sediments and suspended particulates: a review. Hydrobiologia 91-92, 299-313.

Heijerick, D.G., De Schamphelaere, K.A.C., Janssen, C.R., 2002. Prediting acute zinc toxicity for *Daphnia magna* as a function of key water chemistry characterisitics: development and validation of a biotic ligand model. Environ. Toxicol. Chem. 21, 1309-1315.

Heijerick, D.G., De Schamphelaere, K.A.C., Van Sprang, P.A., Janssen, C.R., 2005. Development of a chronic zinc biotic ligand model for *Daphnia magna*. Ecotox. Environ. Safe. 62, 1-10.

Heijerick, D.G., Janssen, C.R., Coen, W.M.D., 2003. The Combined Effects of Hardness, pH, and Dissolved Organic Carbon on the Chronic Toxicity of Zn to *D. magna*: Development of a Surface Response Model. Arch. Environ. Con. Tox. 44, 210-217.

Herbrandson, C., Bradbury, S.P., Swackhamer, D.L., 2003. Influence of suspended solids on acute toxicity of carbofuran to *Daphnia magna*: II. An evaluation of potential interactive mechanisms. Aquat. Toxicol. 63, 343-355.

Heugens, E.H.W., Hendriks, A.J., Dekker, T., van Straalen, N.M., Admiraal, W., 2002. A Review of the Effects of Multiple Stressors on Aquatic Organisms and Analysis of Uncertainty Factors for Use in Risk Assessment. Crit. Rev. Toxicol. 31, 247 - 284.

Hogstrand, C., Verbost, P.M., Bonga, S.E., Wood, C.M., 1996. Mechanisms of zinc uptake in gills of freshwater rainbow trout: interplay with calcium transport. Am J Physiol Regul Integr Comp Physiol 270, R1141-1147.

Hogstrand, C., Wilson, R.W., Polgar, D., Wood, C.M., 1994. Effects of zinc on the kinetics of branchial calcium uptake in freshwater rainbow trout during adaptation to waterborne zinc. J. Exp. Biol. 186, 55-73.

Hollis, L., Burnison, K., Playle, R.C., 1996. Does the age of metal-dissolved organic carbon complexes influence binding of metals to fish gills? Aquat. Toxicol. 35, 253-264.

Hyne, R.V., Pablo, F., Julli, M., Markich, S.J., 2005. Influence of water chemistry on the acute toxicity of copper and zinc to the cladoceran *Ceriodaphnia dubia*. Environ. Toxicol. Chem. 24, 1667-1675.

Kamunde, C., Grosell, M., Higgs, D., Wood, C.M., 2002. Copper metabolism in actively growing rainbow trout (*Oncorhynchus mykiss*): interactions between dietary and waterborne copper uptake. J. Exp. Biol. 205, 279-290.

Kim, S.D., Gu, M.B., Allen, H.E., Cha, D.K., 2001. Physicochemical Factors Affecting the Sensitivity of *Ceriodaphnia dubia* to Copper. Environ. Monit. Assess. 70, 105-116.

Koivisto, S., 1995. Is *Daphnia magna* an ecologically representative zooplankton species in toxicity tests? Environ. Pollut. 90, 263-267.

Kramer, K.J.M., Jak, R.G., van Hattum, B., Hooftman, R.N., Zwolsman, J.J.G., 2004. Copper toxicity in relation to surface water-dissolved organic matter: biological effects to *Daphnia magna*. Environ. Toxicol. Chem. 23, 2971-2980.

Lett, P.F., Farmer, G.J., Beamish, F.W.H., 1976. Effect of copper on some aspects of bioenergetics of rainbow-trout (*Salmo gairdneri*). J. Fish. Res. Board Can. 33, 1335-1342.

Li, J., Lock, R.A.C., Klaren, P.H.M., Swarts, H.G.P., Stekhoven, F.M.A.H.S., Bonga, S.E.W., Flik, G., 1996. Kinetics of Cu2+ inhibition of Na+/K+-ATPase. Toxicol. Lett. 87, 31-38.

Loiterton, B., Sundbom, M., Vrede, T., 2004. Separating physical and physiological effects of temperature on zooplankton feeding rate. Aquat. Sci. 66, 123-129.

Long, K.E., Van Genderen, E.J., Klaine, S.J., 2004. The effects of low hardness and pH on copper toxicity to *Daphnia magna*. Environ. Toxicol. Chem. 23, 72-75.

Lye Koh, H., Hallam, T.G., Ling Lee, H., 1997. Combined effects of environmental and chemical stressors on a model *Daphnia* population. Ecol. Model. 103, 19-32.

Ma, H., Kim, S.D., Allen, H.E., Cha, D.K., 2002. Effect of copper binding by suspended particulated matter on toxicity. Environ. Toxicol. Chem. 21, 710-714.

Ma, H., Kim, S.D., Cha, D.K., Allen, H.E., 1999. Effect of complexation by humic acid on toxicity of copper to *Ceriodaphnia dubia*. Environ. Toxicol. Chem. 18, 828-837.

MacRae, R.K., Smith, D.E., Swoboda-Colberg, N., Meyer, J.S., Bergman, H.L., 1999. Copper binding affinity of rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*) gills: implications for assessing bioavailable metal. Environ. Toxicol. Chem. 18, 1180-1189.

Marr, J.C.A., Lipton, J., Cacela, D., Hansen, J.A., Bergman, H.L., Meyer, J.S., Hogstrand, C., 1996. Relationship between copper exposure duration, tissue copper concentration, and rainbow trout growth. Aquat. Toxicol. 36, 17-30.

Matsuo, A.Y.O., Playle, R.C., Val, A.L., Wood, C.M., 2004. Physiological action of dissolved organic matter in rainbow trout in the presence and absence of copper: Sodium uptake kinetics and unidirectional flux rates in hard and softwater. Aquat. Toxicol. 70, 63-81.

McGeer, J.C., Szebedinszky, C., McDonald, D.G., Wood, C.M., 2000. Effects of chronic sublethal exposure to waterborne Cu, Cd or Zn in rainbow trout. 1: Iono-regulatory disturbance and metabolic costs. Aquat. Toxicol. 50, 231-243.

McGeer, J.C., Szebedinszky, C., McDonald, D.G., Wood, C.M., 2002. The role of dissolved organic carbon in moderating the bioavailability and toxicity of Cu to rainbow trout during chronic waterborne exposure. Comp. Biochem. Phys. C 133, 147-160.

Meems, N., Steinberg, C.E.W., Wiegand, C., 2004. Direct and interacting toxicological effects on the waterflea (Daphnia magna) by natural organic matter, synthetic humic substances and cypermethrin. Sci. Total. Environ. 319, 123-136.

Meyer, J.S., 1999. A Mechanistic Explanation for the ln(LC50) vs ln(Hardness) Adjustment Equation for Metals. Environ. Sci. Technol. 33, 908-912.

Muyssen, B.T.A., De Schamphelaere, K.A.C., Janssen, C.R., 2006. Mechanisms of chronic waterborne Zn toxicity in *Daphnia magna*. Aquat. Toxicol. 77, 393-401.

Muyssen, B.T.A., Janssen, C.R., 2001. Multigeneration zinc acclimation and tolerance in *Daphnia magna*: implications for water-quality guidelines and ecological risk assessment. Environ. Toxicol. Chem. 20, 2053-2060.

Muyssen, B.T.A., Janssen, C.R., 2002. Accumulation and Regulation of Zinc in *Daphnia magna* : Links with Homeostasis and Toxicity. Arch. Environ. Con. Tox. 43, 0492-0496.

Muyssen, B.T.A., Janssen, C.R., 2005. Importance of acclimation to environmentally relevant zinc concentrations on the sensitivity of *Daphnia magna* toward zinc. Environ. Toxicol. Chem. 24, 895-901.

Muyssen, B.T.A., Janssen, C.R., Bossuyt, B.T.A., 2002. Tolerance and acclimation to zinc of field-collected *Daphnia magna* populations. Aquat. Toxicol. 56, 69-79.

Naddy, R.B., Stubblefield, W.A., May, J.R., Tucker, S.A., Hockett, J.R., 2002. The effect of calcium and magnesium ratios on the toxicity of copper to five aquatic species in freshwater. Environ. Toxicol. Chem. 21, 347-352.

Nandini, S., Picazo-Paez, E.A., Sarma, S.S.S., 2007. The combined effects of heavy metals (copper and zinc), temperature and food (*Chlorella vulgaris*) level on the demographic characters of *Moina macrocopa* (Crustacea: Cladocera). J Environ Sci Health A Tox Hazard Subst Environ Eng 42, 1433-1442.

Paquin, P.R., Santore, R.C., Wu, K.B., Kavvadas, C.D., Di Toro, D.M., 2000. The biotic ligand model: a model of the acute toxicity of metals to aquatic life. Environ. Sci. Policy 3, 175-182.

Pelgrom, S.M.G.J., Lock, R.A.C., Balm, P.H.M., Wendelaar Bonga, S.E., 1997. Calcium fluxes in juvenile tilapia, *Oreochromis mossambicus*, exposed to

sublethal waterborne Cd, Cu or mixtures of these metals. Environ. Toxicol. Chem. 16, 770-774.

Perschbacher, P.W., 2005. Temperature effects on acute copper toxicity to juvenile channel catfish *Ictalurus punctatus*. Aquaculture 243, 225-228.

Perschbacher, P.W., Wurts, W.A., 1999. Effects of calcium and magnesium hardness on acute copper toxicity to juvenile channel catfish, *Ictalurus punctatus*. Aquaculture 172, 275-280.

Playle, R.C., Dixon, D.G., Burnison, K., 1993. Copper and cadmium-binding to fish gills - estimate of metal gill stability-constants and modeling of metal accumulation. Can. J. Fish. Aquat. Sci. 50, 2678-2687.

Pyle, G.G., Kamunde, C.N., McDonald, D.G., Wood, C.M., 2003. Dietary sodium inhibits aqueous copper uptake in rainbow trout (*Oncorhynchus mykiss*). J. Exp. Biol. 206, 609-618.

Rainbow, P.S., 1997. Ecophysiology of Trace Metal Uptake in Crustaceans. Estuar. Coast. Shelf. S. 44, 169-175.

Richards, J.G., Curtis, P.J., Burnison, B.K., Playle, R.C., 2001. Effects of natural organic matter on reducing metal toxicity to rainbow trout (*Oncorhynus mykiss*) and on metal biding to their gills. Environ. Toxicol. Chem. 20, 1159-1166.

Ryan, A.C., Van Genderen, E.J., Tomasso, J.R., Klaine, S.J., 2004. Influence of natural organic matter source on copper toxicity to larval fathead minnows (*Pimephales promelas*): implications for the biotic ligand model. Environ. Toxicol. Chem. 23, 1567-1574.

Santore, R.C., Mathew, R., Paquin, P.R., DiToro, D., 2002. Application of the biotic ligand model to predicting zinc toxicity to rainbow trout, fathead minnow, and Daphnia magna. Comp. Biochem. Phys. C 133, 271-285.

Schwartz, M.L., Vigneault, B., 2007. Development and validation of a chronic copper biotic ligand model for *Ceriodaphnia dubia*. Aquat. Toxicol. 84, 247-254.

Sciera, K.L., Isely, J.J., Tomasso, J.R., Klaine, S.J., 2004. Influence of multiple water-quality characteristics on copper toxicity to fathead minnows (*Pimephales promelas*). Environ. Toxicol. Chem. 23, 2900-2905.

Seidl, M.D., Pirow, R., Paul, R.J., 2005. Acclimation of the microcrustacean *Daphnia magna* to warm temperatures is dependent on haemoglobin expression. J. Therm. Biol. 30, 532-544.

Stahl, J.R., Bachman, R.A., Barton, A.L., Clark, J.R., deFur, P.L., Ells, S.J., Pittinger, C.A., Slimak, M.W., Wentsel, R.S., 2001. Risk Management: Ecological Risk-Based Decision-Making. Society of Environmental Toxicology and Chemistry (SETAC), Pensacola, Florida.

Stouthart, X., Haans, J.L.M., Lock, R.A.C., Bonga, S.E.W., 1996. Effects of water pH on copper toxicity to early life stages of the common carp *(Cyprinus carpio)*. Environ. Toxicol. Chem. 15, 376-383.

Taylor, G., Baird D.J., Soares, A.M.V.M., 1998. Surface binding of contaminants by algae: consequences for lethal toxicity and feeding to *Daphnia magna* Straus. Environ. Toxicol. Chem. 17(3), 412-419.

Taylor, L.N., McGeer, J.C., Wood, C.M., McDonald, D.G., 2000. Physiological effects of chronic exposure to rainbow trout (*Oncorhyncus mykiss*) in hard and soft water: evaluation of chronic indicators. Environ. Toxicol. Chem. 19, 2298-2308.

Tipping, E., 1998. Humic Ion-Binding Model VI: An Improved Description of the Interactions of Protons and Metal Ions with Humic Substances. Aquatic Geochemistry 4, 3-47.

USEPA, 1993. Science Advisory Board. Notification of Consultation on Revisions to the Aquatic LifeWater Quality Criteria Guidelines. EPA-SABEPEC-CON-93-006.

Van Genderen, E., Gensemer, R., Smith, C., Santore, R., Ryan, A., 2007. Evaluation of the Biotic Ligand Model relative to other site-specific criteria derivation methods for copper in surface waters with elevated hardness. Aquat. Toxicol. 84, 279-291.

Van Sprang, P.A., Janssen, C.R., 2001. Toxicity identification of metals: development of toxicity identification fingerprints. Environ. Toxicol. Chem. 20, 2604-2610.

Villavicencio, G., Urrestarazu, P., Carvajal, C., De Schamphelaere, K.A.C., Janssen, C.R., Torres, J.C., Rodriguez, P.H., 2005. Biotic ligand model of copper toxicity to daphnids in a range of natural waters in Chile. Environ. Toxicol. Chem. 24, 1287-1299.

Weltens, R., Goossens, R., Van Puymbroeck, S., 2000. Ecotoxicity of Contaminated Suspended Solids for Filter Feeders (*Daphnia magna*). Arch. Environ. Con. Tox. 39, 315-323.

Winner, R.W., 1985. Bioaccumaltion and toxicity of copper as affected by interactions between humic-acid and water hardness. Water Res. 19, 449-455.

Woodling, J., Brinkman, S., Albeke, S., 2002. Acute and chronic toxicity of zinc to the mottled sculpin *Cottus Bairdi*. Environ. Toxicol. Chem. 21, 1922-1926.

Wurts, W.A., Durborrow, R.M., 1992. Interactions of pH, Carbon Dioxide, Alkalinity and Hardness in Fish Ponds Southern Regional Aquaculture Center 464.

Yu, R.-Q., Wang, W.-X., 2002. Kinetic uptake of bioavailable cadmium, selenium, and zinc by *Daphnia magna*. Environ. Toxicol. Chem. 21, 2348-2355.

Yurista, P.M., 1999. A model for temperature correction of size-specific respiration in *Bythotrephes cederstroemi* and *Daphnia middendorffiana*. J. Plankton Res. 21, 721-734.

Zahner, H.M., Vangenderen, E.M., Tomasso, J.R., Klaine, S.J., 2006. Whole-body sodium concentration in larval fathead minnows (*Pimephales promelas*) during and following copper exposure. Environ. Toxicol. Chem. 25, 1635–1639.

3. Evaluation of physic-chemical media characteristics in *Daphnia magna* Straus feeding rates

João A. M. Pedrosa, Fátima T. Jesus, António J.A. Nogueira
CESAM & Departamento de Biologia, Universidade de Aveiro, Portugal
(To be submitted to Environmental Toxicology and Chemistry)

Abstract

The feeding behavior of the aquatic filter-feeder has been widely studied once it has been demonstrated to be a sensitive parameter of the toxic response of organisms exposed to toxicants. However, few data exist about how different water quality characteristics can influence the feeding rates of organisms. In order to explore this lack of information about direct influences of water chemistry we developed a battery of tests where feeding rates of *Daphnia magna* (Branchipoda, Cladocera) were determined along their life-cycle in varying environmental characteristics. Feeding rates of *Daphnia magna* (clone F) were measured for different body lengths and six synthetic media, ranging in hardness and alkalinity. These media were tested at 20º, 25º and 30 º C. Additionally, food concentrations of 1, 2, 4, 7, 10 and 14µg DW/ml were tested for each medium at each temperature. Significant effects of temperature were found, while chemical composition effects were not significant nor the interaction between these parameters. Daphnids also responded to different food concentrations. To the predictive model for feeding rates at a given body length temperature and food concentration effects were discounted.

Keywords: *Daphnia magna*, Feeding rates, Food concentration, Medium composition, Temperature

3.1. Introduction

Industrial effluents and untreated wastes usually end-up in water bodies resulting in serious and adverse impacts to the aquatic ecosystems (Gauthier et al., 2006). It is well documented that heavy metals, such as zinc and copper, exert harmful effects in the aquatic organisms leading to fitness reductions of wild populations and/or indirect foodweb effects (Bianchini et al., 2005).

These metals occur, in the natural aquatic systems, in a variety of physical-chemical forms, or species, which exert different toxic effects at the organisms (Chakoumakos et al., 1979). The free hydrated metal ion (e. g. Cu^{2+}) is, generally, considered the most toxic form but metals can exist under other several forms such as metal complexes with organic and inorganic ligands in dissolved, colloidal or particulate forms (Boeckman and Bidwell, 2006). The relative proportion of these metal forms varies greatly from water to water as a result of water quality characteristics (Santore et al., 2002). For this reason, physical and chemical factors that influence bioavailability have to be included to correctly predict metal toxicity.

As a result of this recognize, recently, has entered in scene the Biotic Ligand Model (BLM) (de Schamphelaere and Janssen, 2002; Santore et al., 2002). The major strength of these models results from the consideration that metal bioavailability to aquatic organisms is the result of the interaction between biotic and abiotic (water characteristics) factors. It inserts the biotic ligands into aquatic chemistry programs that run using thermodynamic equilibrium stability constants (Playle, 1998) and account the chemical speciation of several ions present in solution. These models, initially applied to fish species (Santore et al., 2002), have been introduced to predict acute (de Schamphelaere and Janssen, 2002) and chronic (Heijerick et al., 2005) toxic responses in cladoceran species.

However, cladoceran tests (such as the ones designed to build BLMs) are determined following standard guideline procedures to evaluate the toxic effect on the organisms, which provide little information about the cause of action of the compounds (e. g. OECD, 2004). The diagnostic of the toxic effect is done by test designs predicting lethal (acute tests of 48h) and chronic (over one or more generations) effects on the organisms. In fact, current test protocols based on lethality and reproduction parameters alone may be underprotective, once they not give a complete analysis of the toxic effect of a certain pollutant (Orchard et al., 2000). Chronic tests are unable to discriminate if sub-lethal effects on reproduction are mediated by supply- and/or demand-side effects and thus they also are unable to predict long-term toxic effects on phytoplankton communities due to their effect on grazing (Jak et al., 1996). The

toxic effect of a pollutant may include reduced resource acquisition (supply-side effects, (McWilliam and Baird, 2002)) and thus reduce offspring production and/or increase age at first reproduction (Barata, 1998). Once the dominant mode of action of a toxic substance has been determined it should be possible to estimate potential effects to the population by use of individual energetic models (Schindler, 1968; Gurney et al., 1990).

Consequently, it is of major importance to determine possible supply effects on organisms as a result of pollutant expositions. Cladocerans' feeding rates, defined by (Gauld, 1951) as the amount of food which the animal can obtain in a given time, constitute a sensitive indicator of toxicity. The big advantages of using feeding rates as endpoint are: its rapid assessment and its physiological and ecological relevance (McWilliam and Baird, 2002). Indeed, cladocerans are important elements of the aquatic communities once they feed phytoplankton and consist in prey items for predatory zooplankton and fish. A change in cladoceran populations due to a reduction in feeding rate can be important, since the grazing pressure of cladocerans on algae can be a potential factor structuring plankton communities (Orchard et al., 2000). Reduction in algal ingestion rates of cladocerans can traduce in decreases in developmental rate, growth rate, fecundity and survival rates and, in natural conditions, these mechanisms may lead to changes in the relative abundance of both cladocerans and phytoplankton communities (Kirk, 1991).

The aim of this study was to develop mathematical relationships predicting feeding rates as a critical endpoint to risk assessment using the most commonly used cladoceran *D. magna* Straus as test organism. In this paper we will discuss the influence that natural physic and chemical characteristics can exert in the feeding response of *D. magna*. For this purpose, organisms' feeding rates will be tested at different temperatures and media composition, with varying alkalinity, hardness, pH and major ions content. The ultimate objective of this paper is find the simplest model that can account for the feeding behavior of *Daphnia magna* exposed to a variety of water characteristics.

The "temperature" parameter will also be tested because while the effects of water chemical characteristics have been intensively studied, relatively lower importance has been given to temperature effects. For example,

in the BLMs, this aspect is only considered to the calculations of the thermodynamic equilibrium constants (e.g. Di Toro et al., 2001). The laboratory assays are conducted at constant and favourable temperature conditions (mostly at 20º C) (e.g. OECD, 2004). However, it is well known that temperature varies greatly according to diary and seasonal cycles (Mckee and Ebert, 1996). Consequently, once the biggest part of the aquatic organisms is ectothermic, an interaction between temperature and toxicants is also expected (Goss, 1980). In fact, temperature may influence toxicokinetics of compounds by affecting metabolic rate or locomotory and feeding activity of an organism, thus affecting uptake, elimination, and detoxication rates (Heugens et al., 2002). At the thermal tolerance limits, exposure to toxicants can enlarge adverse thermal effects (Nandini et al., 2007).

3.2. Materials and Methods

3.2.1. Culture Maintenance

Females of *D. magna* clone F, a laboratory well-established clone, were maintained in individual cultures of 100 ml ASTM hard water and fed daily with the green algae *C. vulgaris* at a concentration of 7µg algae dry weight per ml of medium (µg DW/ml). The culture medium was renewed every other day and 6ml of seaweed extract per litre of culture medium was added. Daphnids were kept in climatic chambers at 20±1ºC under a photoperiod of 16:8 hours light: darkness.

The food resource, *Chlorella vulgaris,* was cultured in MBL medium at a constant temperature of 20±1º C, with continuous aeration and light under a period of 7 to 10 days (till reach the stationary growth phase). After it, alga were centrifuged, the supernatant was discharged and pellet collected, stored for a maximum of 7 days and used to feed the daphnids.

3.2.2. Acclimatization of test organisms

Animals were maintained individually in 100ml ASTM hard water till the moment they started developing eggs in the brood chamber. When reached that

point, they were removed and randomly distributed to individual flasks containing one of the six US-EPA synthetic inorganic media (Table 3.1), prepared with ultrapure water (Milli-Q; 18MΩcm^{-1} resistivity) and laboratory degree reagents. These testing media varied in alkalinity (from low to very high alkalinity) and hardness (from soft to very hard waters). Simultaneously, animals were also acclimatized to the test temperatures (20º, 25º and 30º C).

Media were renewed three times a week and seaweed extract (concentration of 6 ml/L) was also addicted. Organisms were fed daily with 7µg DW/ml *C. vulgaris.*

The experimental daphnids, organisms from third to fifth broods, were collected and transferred to the same physic-chemical conditions of their mothers. Then, the organisms were led to grow till the moment they were at the instar stage which the test would be conducted.

The presence of moults in the beakers containing experimental daphnids was verified twice a day, to make sure about organisms' instar stage.

Table 3.1– Salt composition, in mg/L, of the tested media and alkalinity and hardness classification

	NaHCO3 (mg/L)	MgSO4.7H2O (mg/L)	KCl (mg/L)	CaSO4.2H2O (mg/L)	Alkalinity	Hardness
Medium 1	48,00	30,00	2,00	30,00	Low	Soft
Medium 3	96,00	60,00	4,00	60,00	Moderate	Moderate
Medium 4	192,00	60,00	8,00	60,00	High	Moderate
Medium 5	192,00	120,00	8,00	120,00	High	Hard
Medium 7	48,00	120,00	8,00	120,00	Low	Hard
Medium 10	384,00	240,00	16,00	240,00	Very high	Very hard

3.2.3. Feeding tests

The experimental design consisted of 10 plastic containers per medium filled with 50 ml of culture medium and food was added at a predefined concentrations. Five flasks were replicates (with organisms), three were blanks (with no animal addiction) and two remaining flasks were used for initial determinations of pH and conductivity. The feeding tests were conducted at

darkness with the aim of reducing algae growth at the beakers. The feeding tests occurred in the same physic-chemical conditions of daphnids' growth. At each feeding assay, the animals were all at the same instar stage.

The absorbances were measured at 440nm. Blank absorbance was measured at the beginning and at the end of the test and the absorbance of the replicates was measured only at the end.

The exposure periods were short, varying between 3 and 6 hours, to minimize food deposition in the bottom of the flasks. The number of organisms per replicate container also varied (between 2 and 7) according to organism's body length and food concentration to ensure comparable food depletion in all treatments over the exposure period.

After the feeding experiments the presence of moults at each replicate was verified. When moults were found, the replicate was excluded. After that, daphnids were collected with a 3ml plastic pipette to glass jars containing the same media and allowed to grow till released the next moult. When it happened, animals were discharged and moults were collected to determine body lengths. Body length was determined for all individuals used in the tests .

The feeding rates of *Daphnia magna* (clone F) per organism was calculated for each replicate using a simplified version (3.1) of Gauld's equation (Gauld, 1951):

$$F = \frac{V(C_i - C_f)}{nT} \qquad \textbf{(3.1)}$$

Where F is the feeding rate (measured in µg algae dry weight per organism per hour), V is the sample volume (in ml), C_i (in µg DW *C. vulgaris*/ml) is the blank absorbance (at 440nm) at the end of the experiment, C_f (in µg DW *C. vulgaris*/ml) is the replicate absorbance (at 440nm) at the end of the experiment, *n* is the number of organisms per beaker and *T* is the exposure time (in hours).

3.2.3.1. Feeding tests at different algae concentrations

The experiments were conducted with fourth instar stage *D. magna* females during an experimental period of 4 hours.

Groups of three organisms per flask were tested at 1, 2, 4, 7 and 10 µg DW/ml and 5 organisms per flask were tested at 14 µg DW/ml.

3.2.3.2. Feeding tests at different body length

The feeding experiments at different body lengths (BL) had little variations in the experimental procedures. These experiments were conducted at 7µg DW/ml *C. vulgaris.* The number of organisms per flask and the exposition time at the experiments varied according to daphnids' body length. Thus, the feeding experiments conducted with organisms at second instar stage had an exposition period of 6 hours; organisms bigger than 4mm had 3 hours of exposition time; the remaining experiments, conducted with organisms between third instar stage and organisms smaller than 4mm, were performed during four hours.

In terms of number of organisms, the feeding experiments performed with daphnids at the second instar had seven organisms per flask, in third instar feeding experiments we put five organisms per flask, in fourth and five instar feeding experiments we put three organisms per flasks and to feeding assays performed with bigger daphnids we put two organisms per flask.

3.2.4. Chemical analysis

Water hardness and alkalinity were determined according to the standard methods, using three replicates per medium for each analysis.

For major ion determination three extra flasks were also filled and seaweed extract (6ml/l) and algae (7 µg DW/ml) were addicted to the medium. Samples were filtered through a 0,45µm filter to recoil only the dissolved phase. After that, a certain volume per sample was recoiled to chloride and sulphate determinations using the HACH-Kit, method 8113 and method 8051, respectively.

The remaining filtered volumes were acidified (pH<1) to posterior analysis of calcium, magnesium, potassium and sodium. These cations were analyzed using spectrometer.

3.2.5. Statistical analysis

The feeding rates obtained at the reference algae concentration (7µg *C. vulgaris* DW/ml) were used to estimate the effects of chemical and physical variations. The influence of chemical and physical composition was determined using a standard ANCOVA analysis (Zar, 1998). The effects of medium type and temperature were assessed with a factorial ANOVA (Minitab™ Release 14). Based on these tests, the parameters that significantly influenced the feeding rates were incorporated in the predictive model for feeding rates of *Daphnia magna* (clone F).

3.3. Results

The statistical analysis is presented on Table 3.2. The analysis of transformed data revealed that chemical composition of the water is of lower importance in the feeding response of daphnids. At 20º, 25º and 30º C the ANCOVA test did not show significant differences between the six media tested. However, significant differences were obtained for each individual medium when the effect of temperature variation was tested. Medium 1 showed significant differences for coincidental regression of the feeding response, log transformed, at 20º, 25º and 30º C but did not show significant differences in the slope nor in the interception of the regression. Medium 3 and 4 showed significant differences for the test comparison of coincidental regressions and also significant differences between slopes. Medium 5, 7 and 10 demonstrated significant differences between the regressions, slopes and interceptions.

Table 3.2– Results of the statistical ANCOVA testing the feeding rates log transformed of individual *D. magna* against the body length of the organisms. The effects of the factors "exposure medium" and "temperature" are presented here.

Null Hypothesis (H_0)	Coincidental regression lines		Slopes comparison		Elevations comparison	
	F values	P	F values	P	F values	P
Feeding rates tested at medium 1 do not vary according to temperature	$F_{[4,80]}$=3,29 *	0,015	$F_{[2,80]}$=1,92	0,150	-	-
Feeding rates tested at medium 3 do not vary according to temperature	$F_{[4,94]}$=8,54 *	0,000	$F_{[2,94]}$=15,82 *	0,000	$F_{[2,96]}$=0,96	0,387
Feeding rates tested at medium 4 do not vary according to temperature	$F_{[4,90]}$=8,08 *	0,000	$F_{[2,90]}$=13,24 *	0,000	$F_{[2,92]}$=2,30	0,106
Feeding rates tested at medium 5 do not vary according to temperature	$F_{[4,91]}$=13,25 *	0,000	$F_{[2,91]}$=20,55 *	0,000	$F_{[2,93]}$=4,19	0,018
Feeding rates tested at medium 7 do not vary according to temperature	$F_{[4,93]}$=17,37 *	0,000	$F_{[2,93]}$=24,06 *	0,000	$F_{[2,95]}$=7,19 *	0,001
Feeding rates tested at medium 10 do not vary according to temperature	$F_{[4,85]}$=20,15 *	0,000	$F_{[2,85]}$=29,18 *	0,000	$F_{[2,87]}$=6,74 *	0,002
At 20º C, feeding rates tested do not vary according to media	$F_{[10,154]}$=1,37	0,201	-	-	-	-
At 25º C, feeding rates tested do not vary according to media	$F_{[10,169]}$=1,67	0,092	-	-	-	-
At 30º C, feeding rates tested do not vary according to media	$F_{[10,210]}$=1,202	0,291	-	-	-	-

3.3.1. Body length

The tests executed with the 6 artificial media revealed great differences between the feeding rates obtained. The global feeding rates, with 7µg DW/ml of algae supply, varied between 0,34 and 27,38 µg *C. vulgaris* DW per

organism per hour. These results, at a first instance, show that feeding rates varied with the body length.

As expected, feeding rates varied with the body length at the three tested temperatures. At 20º C the feeding rates ranged between 0,81 and 21,67 µg DW·organism^{-1}·hour^{-1} and the organisms ranged between 1,19 and 4,25mm. At 25º C the feeding rates were within 0,57 and 27,37, with the organisms ranging between 1,09 and 4,25 mm. At 30º C, the feeding rates were inside the interval 0,34 – 17,10, and the organisms varied between 0,82 and 3,29mm.

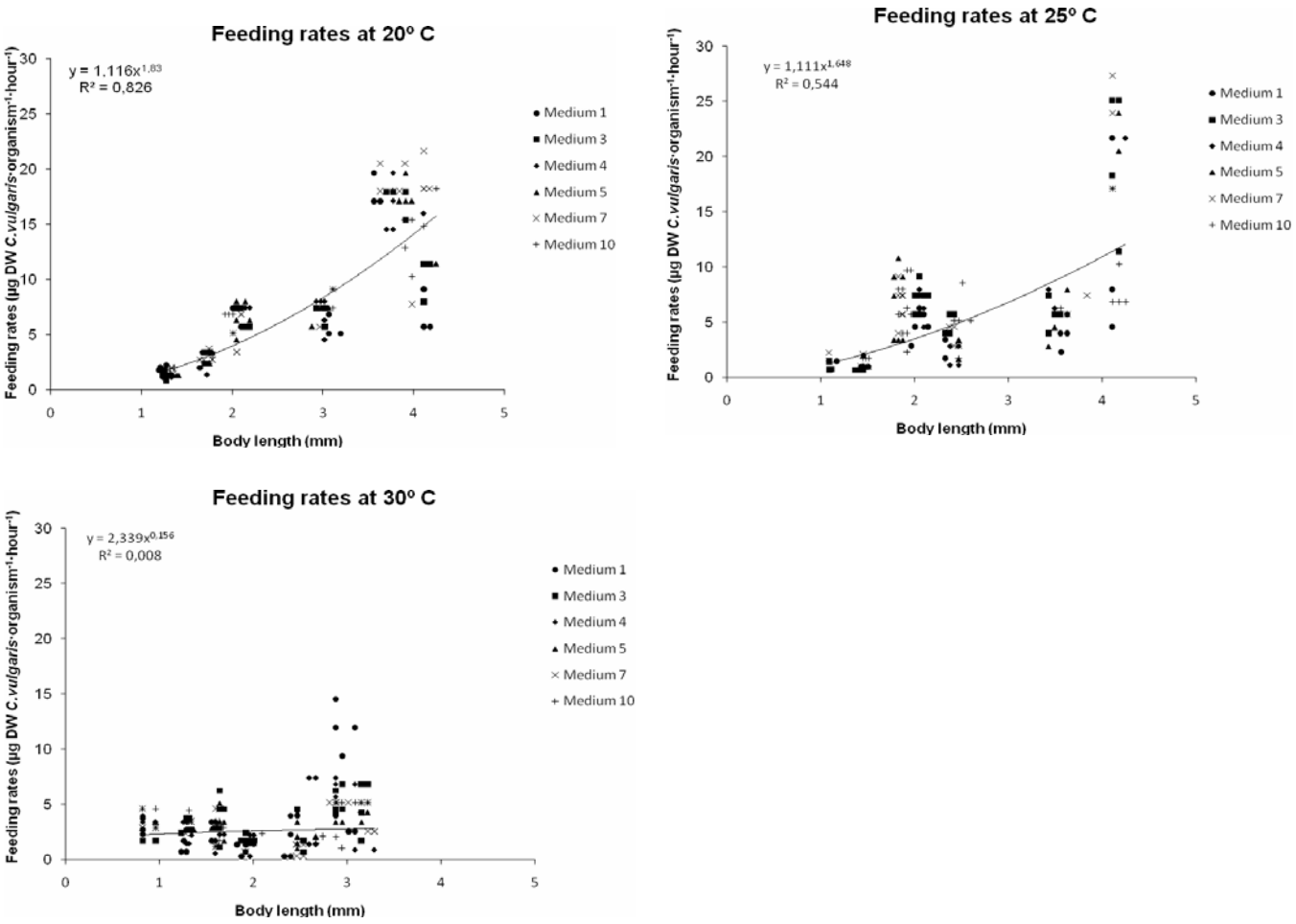

Figure 3.1 – General regression lines for 20º, 25º and 30º C plotting log Feeding rates per organism per hour *versus* log Body length performed at constant food concentration of 7 µg *C. vulgaris* DW/ml

3.3.2. Temperature effects

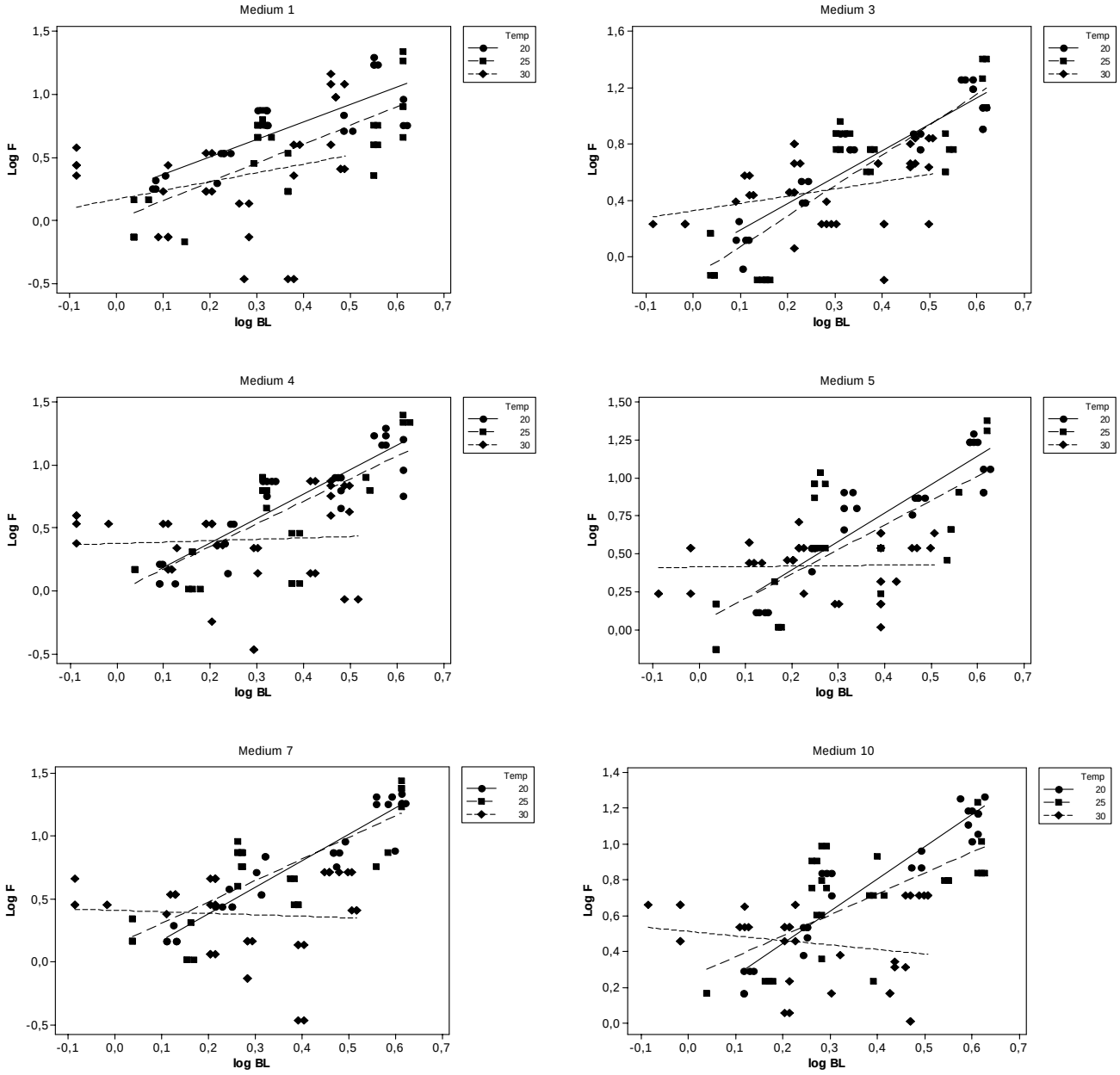

Figure 3.2 – Regression lines for each medium at 20º, 25º and 30º C. Log Feeding rates per organism per hour (log F) is plotted against log Body length (log BL)

The feeding response for each medium at the three temperatures tested was compared. For each medium significant difference in feeding rates per temperature was observed. In a general way, daphnids fed at 7µg DW/ml

showed higher feeding rates at a temperature of 25±1º C and the lowest feeding rates were obtained at 30º±1º C. At 30º C it seems that organisms ingest little portions of food when compared with feeding rates of organisms at 20º and 25º C.

This phenomenon is shown by the slope of the regression lines (Figure 3.2). Additionally, at 30º C, the variability between replicates highly increases with the increase of body length, resulting in little correlation. Moreover, we could not test organisms with 4mm. At this temperature organisms grew less and they could not reach big body lengths, succumbing before reaching 4mm.

3.3.3. Chemical composition effects

The chemical composition of the testing waters used for the feeding assays, at each temperature, is reported in Table 3.3. It is important to note that seaweed extract added to the media resulted in higher concentrations of major cations.

Feeding rates log transformed, conducted at constant food concentration, were regretted against the logarithm of body length. The regressions, the slope and the interception of each medium were compared. At 20º C, 25º C and 30º C the feeding rates seem not to be affected by chemical variations, once ANCOVA analysis was not significant ($p > 0.05$). Changes in hardness, alkalinity, pH, conductivity and major ion concentrations seem not to significantly affect daphnids' feeding rates.

The common regression line explains 82,6%, 54,4%, 0,8% of response variation, respectively at 20º, 25º and 30º C. Feeding rates seem not to be affected by chemical variations. Additionally, the general linear regression analysis of variance, testing the interaction between medium and temperature, was also not significant ($p > 0.05$).

Table 3.3 – Mean values of the physic-chemical characteristics of the US-EPA synthetic media used to *D. magna* Straus (clone F) feeding assays

Medium	T (º C)	pH	Conduct. (µS/s)	Alcalinity (mg $CaCO_3$ L^{-1})	Hardness (mg $CaCO_3$ L^{-1})	Na* (mg/l)	Ca* (mg/l)	Mg* (mg/l)	K* (mg/l)	SO_4^{2-}* (mg/l)	Cl^-* (mg/l)
1	20º C	7,92	160,10	30,86	46,19	15,36	7,37	6,69	3,89	57,67	3,28
	25º C	7,94	156,02	28,87	42,68	15,54	7,46	6,77	3,94	57,00	3,32
	30º C	7,93	163,25	28,00	42,37	17,83	8,56	7,77	4,52	49,67	2,23
3	20º C	8,15	284,40	57,29	89,71	29,83	14,82	12,81	4,94	100,33	4,17
	25º C	8,21	286,00	55,07	82,64	28,69	14,25	12,32	4,75	104,33	4,01
	30º C	8,17	297,00	53,73	82,92	32,77	16,28	14,07	5,43	91,33	2,93
4	20º C	8,36	386,00	109,65	88,17	57,17	14,70	12,40	7,00	102,67	6,39
	25º C	8,36	386,33	107,17	84,07	54,35	13,97	11,79	6,65	108,00	6,07
	30º C	8,33	402,25	104,93	82,01	62,44	16,06	13,54	7,65	94,00	4,73
5	20º C	8,28	539,20	112,35	171,96	56,56	29,66	24,50	6,87	190,83	6,15
	25º C	8,30	538,00	105,83	164,64	53,97	28,30	23,38	6,56	200,00	5,87
	30º C	8,29	554,00	106,87	165,45	58,87	30,87	25,50	7,15	183,33	4,30
7	20º C	7,86	403,80	32,43	169,90	20,17	29,83	24,77	7,07	190,83	5,83
	25º C	7,92	402,00	28,93	171,07	19,17	28,34	23,54	6,72	200,83	5,54
	30º C	7,88	414,00	29,20	165,71	20,81	30,77	25,55	7,29	185,00	4,27
10	20º C	8,24	998,80	227,56	336,23	112,11	68,24	49,33	11,70	376,67	11,87
	25º C	8,26	991,83	269,40	336,27	108,74	66,19	47,85	11,35	388,33	11,53
	30º C	8,15	1005,75	213,47	333,90	112,11	68,24	49,33	11,70	376,67	7,13

* Concentrations of dissolved ions

3.3.4. Food Concentration influence

At the three tested temperatures the organisms' feeding rates responded to the available food concentration, showing higher feeding rates at higher food concentrations and lower feeding rates at lower food concentrations.

3.3.5. The predictive model

Statistical analysis demonstrated that temperature and food concentration had significant effects on the feeding rates of an organism of a certain body length. Chemical variations only caused minor changes in daphnids' feeding rates and these effects were not significant ($p>0.05$). By this reason, possible hardness, alkalinity, pH conductivity and major ions effects were not included on our predictive model.

3.3.5.1. Body Length (BL) influence

The allometric relationship for ingestion indicates that an S-shaped growth curve will be produced when food supplies are adequate (Gurney et al., 1990). The common S-shaped is due to decreases in growth increments in adults that accelerate because maintenance rates increase with body size at a more than linear rate.

According to this statement, data obtained in the feeding assays with *D. magna* (clone F) were adjusted to a global allometric relationship, which has been observed for many *Daphnia* species, predicting the maximum feeding rates (F_{max}) at a certain body length

$$F_{\max} = F_{coef} \times BL^{i} \qquad \textbf{(3.2)}$$

Where F_{max} is the feeding rate (µg *C. vulgaris* dry weight per organism per hour), F_{coef} is the feeding coefficient and i the feeding index (F_{ind}). The X values (BL) correspond to the body length (mm). For each temperature, a global equation, including data obtained in the six media were regretted (Figure 3.1).

After that we ameliorated the sum of squares (using the solver function of Microsft Excel®) of the global equation of each temperature by adjusting the values of the obtained feeding coefficient (F_{coef}) and the feeding index (F_{ind}).

3.3.5.2. Food Concentration influence

Data obtained from the experiments performed at different food concentrations, using individuals in the same instar, were used to determine the F_{half}, the algae concentration which reduces 50% the feeding rates.

We included, also, the food concentration for daphnids on our model, because it is well-known that daphnids respond to food availability in medium. A literature example of this relationship is given by the work of (Schindler, 1968) who found significant differences in *Daphnia magna* feeding rates when exposed to food concentrations ranging from 1 to 10 mg/l. As a result of that we adjusted the feeding rate of organism to the food concentration, addicting the feeding half (F_{half}) to the global model.

At a given size, daphnid feeding rate (*FR*) follows a type 2 functional response that has been well described by Holling (1959, 1966). Several authors successfully applied it to *Daphnia* (e.g. de Mott, 1982; Porter et al., 1982) by the standard Michaelis-Menten function:

$$FR = F_{\max} \times \frac{C}{C + F_{half}} \qquad \textbf{(3.3)}$$

where F_{max} is the saturation feeding rate (calculated at the allometric relationship between feeding rate and body length), *C* is the food concentration and F_{half} (the half-saturation constant) is the food concentration at which intake reaches one half of the asymptotic maximum rate (Schindler, 1968). The F_{max} is dependent of daphnid's body length; however, F_{half} seems not to vary with the size of the individual within a particular species (Gurney et al., 1990).

Seed values for F_{half} can be obtained by applying linear regression to the linearized form of (3.1) the relationship, to an equation type: $y = a + bx$

$$\frac{1}{FR} = \frac{1}{F_{\max}} + \frac{F_{Half}}{F_{\max}} \bullet \frac{1}{C} \qquad \textbf{(3.4)}$$

where F_{half} corresponds to the slope of the graphic 1/FR *versus* 1/C. In this graphic we used data obtained with the tests performed at different food concentrations.

After obtaining the values for the three constants calculated we included this parameter in the general model, obtaining the feeding rates per organism adjusted to the food concentration. After that, we ran the Solver plugin of MS Excel® to find the set of parameters (F_{half}, F_{coef} and F_{ind}) that would minimize the sum of squares of the global model.

3.3.5.3. Temperature influence

Once, temperature significantly affected daphnids' feeding rates, this parameter was included in the model. The model equation applied to "temperature" parameter was a maximum equation, type

$$F_{coef} = k_1 e^{k_2 x}(1 - k_3 e^{k_4 x}) \qquad \textbf{(3.5)}$$

This equation produces a maximum using the product of two functions: one increasing, the other decreasing with increasing *x* values (Haefner, 2005).

Temperature effect was predicted using the feeding coefficients of the allometric equations for each temperature. The maximum equation constants (k_1, k_2, k_3 and k_4) were determined to best fit the feeding coefficients (F_{coef}) obtained at 20º, 25º and 30º C.

The curve that predicts temperature effects is given by the following mathematical equation:

$$F_{coef} = k_1 \times e^{k_2 \times Temp}(1 - k_3 \times e^{k_4 \times Temp}) \qquad \textbf{(3.6)}$$

The maximum type equation obtained resulted in the maximum feeding rate at 24ºC and predicts that between 32º and 33º C daphnids will die.

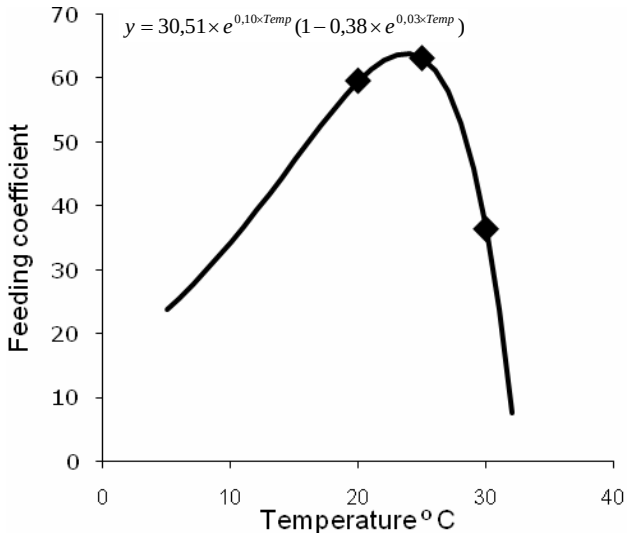

Figure 3.3– Temperature curve for D. magna (clone F) feeding rates

3.3.5.4. Global model

The basic model, integrates three variables: body length, food concentration and temperature.

The mathematical equation that integrates this interaction is given by:

$$F = k_1 \times e^{k_2 \times Temp}(1 - k_3 \times e^{k_4 \times Temp}) \times BL^{\beta} \times \frac{k_h}{k_h + [Food]} \qquad \textbf{(3.7)}$$

Substituting the constants by numbers, we obtained the following general equation predicting the feeding rates (µg DW *C. vulgaris* organism^{-1}.hour^{-1}):

$$F = 30.51 \times e^{0.10 \times Temp}\left(1 - 0.38 \times e^{0.03 \times Temp}\right) \times BL^{1.19} \times \frac{1.49}{1.49 + [Food]} \qquad \textbf{(3.8)}$$

On the following graphics, the Feeding rates expected by the model are scattered against the obtained Feeding rates (Figure 3.4). Additionally, residuals are also scattered (Figure 3.5) according to the tested variables: body length, temperature, medium and food concentration.

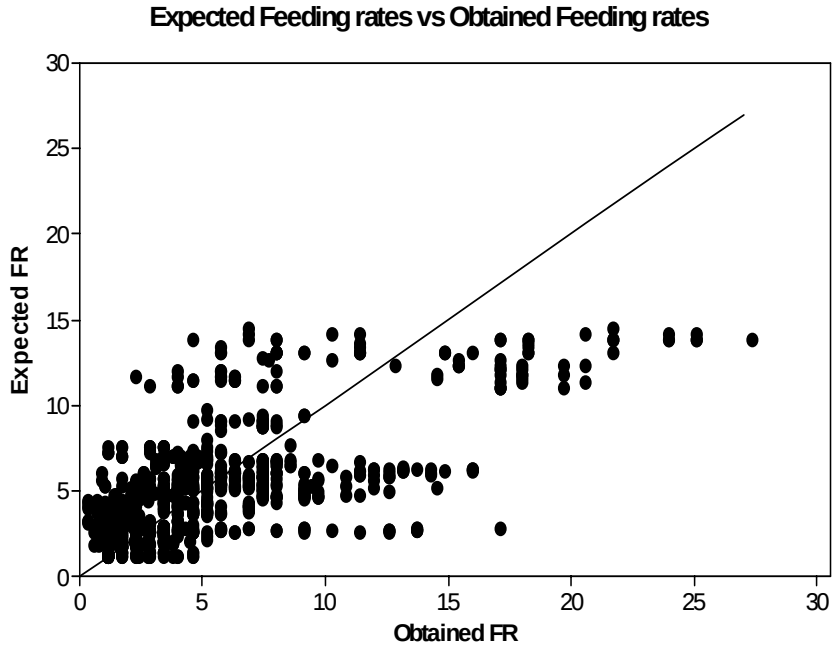

Figure 3.4–Expected Feeding rates (µg Dry Weight *C. vulgaris* per organism per hour) by the model plotted against Obtained Feeding rates

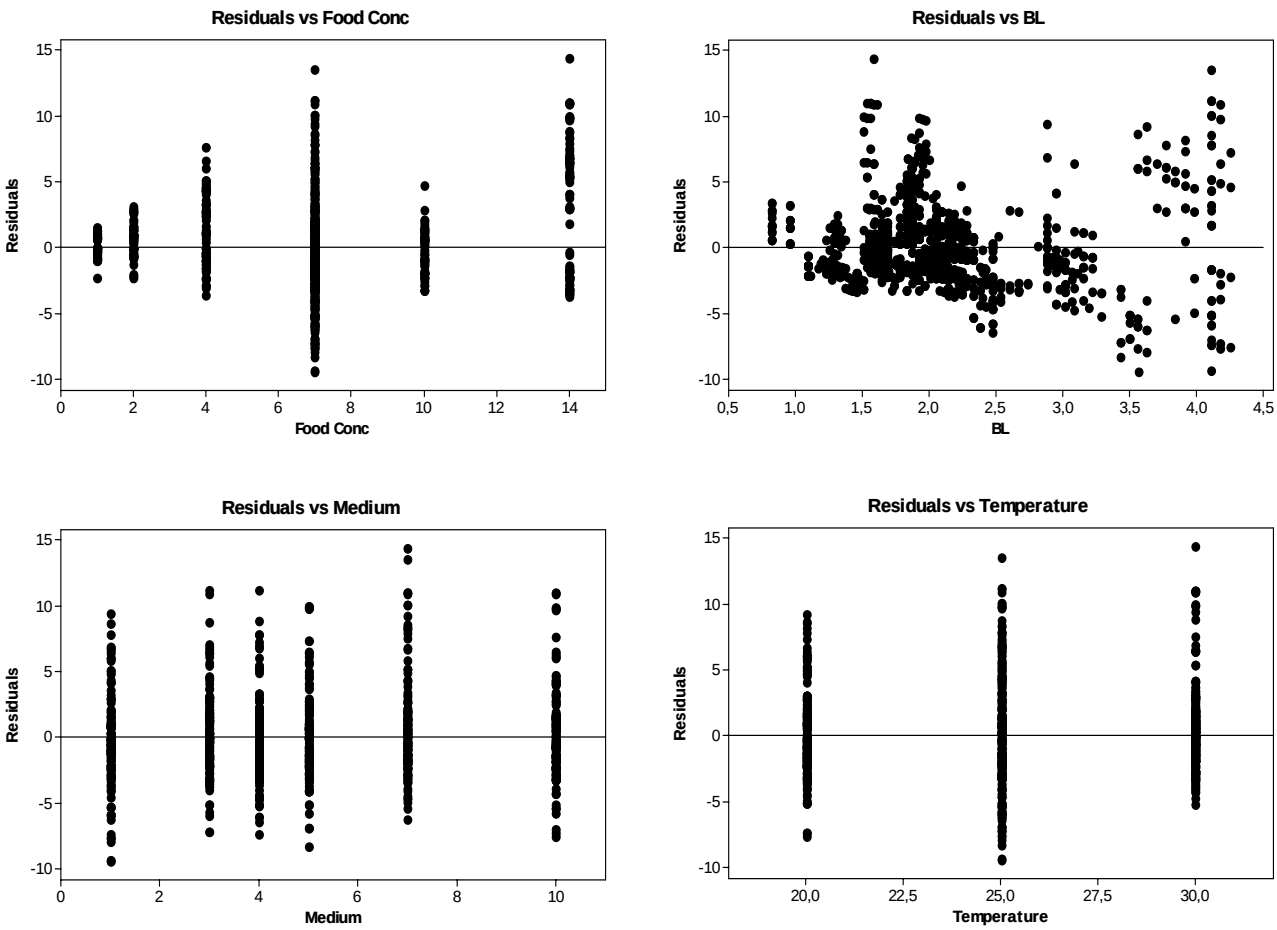

Figure 3.5– Feeding rates' residuals (obtained less expected) according to Food concentration (µg DW/ml), Body Length (BL in mm), Medium and Temperature (º C)

3.4. Discussion

The endpoint "feeding rates per daphnid" was tested along the developmental stages of *Daphnia magna*, where multiple chemical and physical water characteristics were varied. The feeding rates were performed with the green algae *Chlorella vulgaris* because of its sphericity and little variation in size and morphology within cultures. The statistical analysis of our data showed that the main factors affecting the organisms' feeding rates were the body length, temperature and food concentration.

Indeed, feeding rates (and therefore growth rates), are directly linked to temperature sensitive metabolic rates in poikilothermic organisms like *Daphnia*, that also depend on ambient food concentration and quality, existing a strong

interactive influence between these two environmental parameters (Mckee and Ebert, 1996). In this context, (Perrin, 1988) showed that temperature, food concentration and the interaction between these two parameters can significantly affect mean mother weight cladocerans, clutch number and offspring body length (with smaller offspring at higher temperatures).

3.4.1. Preliminary tests

In preliminary tests (data not showed), conducted at 20º C, we varied the number of organisms and the volume per flask. Different volumes (50 and 100ml), number of fourth instar stage daphnids per beaker (three and five daphnids) and exposition time (exposures of four, six and eight hours) were tested. The results demonstrated that the combination resulting in little variation between replicates was three organisms per flask with 50ml of medium during four hours of exposition. So this combination was our basis to the feeding assays and just few adjustments were made aiming to consider different ingestions resulting from body length and food concentration variations. The number of organisms and the exposition time had slight differences, in order to have similar reductions of the initial algae content of the beakers. This reduction was estimated to be between 15 and 25% of the initial algae content.

3.4.2. Body length

The feeding rates were higher at bigger organisms and lower at smaller organisms, responding to needs in terms of energy requirements to their metabolic and anabolic necessities. However, this relationship between feeding rates and body length is not linear (Gurney et al., 1990). The amount of energy available for growth increases during the first juvenile instars, but then it starts to decline as a consequence of energy allocation to reproduction. The growth increments are consecutively decreased as a result of increased maintenance rates (Gurney et al., 1990). Juveniles spend approximately 55% of net assimilation on growth (Tessier et al. 1983), whereas adults spend as much as 80% of assimilated energy on reproduction (Hallam et al., 1990). Consequently, the relationship for ingestion indicates an allometric growth curve when food

supplies are adequate (Gurney et al., 1990). Actually, this comparison between feeding rates of organisms of different body length can only be done because the maximal appendage beat has been observed not to differ between organisms of different body size (Plath, 1998).

3.4.3. Temperature effects

Usually, the toxicity tests in laboratory are performed at 20º C while in real field situations it does not occur. In fact, temperature is highly variable, depending of season and climate (Mckee and Ebert, 1996). Additionally to these natural variations, it is now recognized that climate is changing in earth due to pollution. Recent years led to fluctuations in the temperature regime of many ecosystems which, ultimately, can alter the biology of ectothermic animals (Pörtner, 2001). These aspects are of major relevance and such considerations should be taken into account in the predictive model.

Our results showed that filtering rates initially increased with temperature up to their respective optimal temperatures and then decreased with further temperature increase, what is in accordance with the literature reported above. The determined feeding rates were highest at 25º C and the lowest feeding rates were obtained at 30ºC. In fact, at 30º C daphnids ingested lower amounts of food and they did not even reach bigger body lengths.

Several authors have identified that individual metabolism and growth of *Daphnia* are indeed highly dependent on temperature (Lye Koh et al., 1997). The temperature increase has been observed to increase metabolic activity and energy requirements of *D. magna* (Weetman and Atkinson, 2004). At high temperatures, the moult processes are very rapid, the organisms moult very often and organisms ingest few quantities of food (Mckee and Ebert, 1996). Consequently, temperature increases result in adverse impacts in reproduction (Goss and Bunting, 1980) and reduced life span and body size of *D. magna* (Weetman and Atkinson, 2004).

Our model predicted an optimum temperature value of 24º C (Figure 3.3), which is in accordance to (McMahon, 1965). Additionally, (Kersting and van der Leeuw, 1976) obtained maximal feeding rates between 22º and 26º C while a reduction of temperature from 22º to 10º C was accompanied by a

decrease in feeding rates in their experiments. The maximum temperature predicted by our model was between 32º and 33º C. This maximum value is lower than the one related by (Lye Koh et al., 1997) that predicted an upper temperature limit for *D. magna* of 35º C, the temperature at which all die. However, the same author discussed that *Daphnia* populations would possibly not survive for temperature exceeding 30,6º C.

3.4.4. Food concentration

This parameter is one of the most important environmental variables. Food supply is highly variable in aquatic habitats, fluctuating during the day and station of the year (Gliwicz and Guisande, 1992). Consequently, different generations of planktonic herbivores must often live at different food levels. The uptake of food is proportional to the food concentration up to a critical concentration where the food uptake is maintained constant (Kersting, 1978).

The organisms responded to the food concentration variations from 1 to 14 µg/ml. These results corroborate the ones obtained by (Schindler, 1968) who, previously, had obtained significant feeding differences in *D. magna* exposed to medium with variable concentration of *Chlorella vulgaris* from 1 to 10 mg/L.

Food concentration can seriously affect daphnid organisms' health. Low food levels can result in harmful effects on birth-rate in three different ways: reducing the female size, decreasing the batch size for each size class of females and slowing down the moulting frequency (Vijverberg, 1976; Nogueira, 2004). These damaging effects can be probably associated to low energy reserves in organisms (Mckee and Ebert, 1996).

3.4.5. Chemical variation

Alkalinity and hardness variations, as a result of different ion composition (sodium, potassium, calcium, chloride and sulphate concentration), produced variations in pH values and conductivity also. However, possible effects of water hardness, alkalinity, pH, conductivity and major cation concentration could not explain the remaining variability between organisms' feeding rates along their life cycle.

From our knowledge, it seems that the impact of different water chemical characteristics, in free metal situations, has not been experimentally tested. However, some reports exist about isolated effects of calcium and pH in daphnid's health.

Calcium has been reported as an important water parameter in daphnid's distribution. Indeed, invertebrates can be limited by low Ca in extreme softwater localities. Calcium concentrations between 0.1 – 0.5 mg Ca/L can result in significant mortality of the organisms. Calcium levels between 0.5 – 1.0 mg Ca/L can result in decreased body lengths of the organisms. And, finally, age-specific egg production can be strongly reduced at Ca concentrations <10 mg Ca/L (Alstad et al., 1999). These effects can be due to indirect effects of reduced growth rates or even due to higher energetic costs associated with calcium uptake in Ca-poor medium (Hessen et al., 2000). Additionally, these effects can be increased with the body length increase, once large-bodied daphnids have especially high Ca demands (Wærvagen et al., 2002).

In fact, in our experiments, medium 1 had Ca concentrations lower than 10mg Ca/L. However, it does not contradict our finds because it seems that this sensitivity to low calcium levels is not attributed to "supply-side effects" but to increased metabolic costs.

Low pH can also be prejudicial to daphnids due to sodium disruptions (Glover and Wood, 2005). This fact can explain why freshwater organisms, such as cladocerans are so sensitive to aquatic acidification. The six US-EPA media tested at this paper will not allow us to determine the pH influence at copper and zinc toxicity, once pH was maintained between 7,86 and 8,36 (at 20º C). So a test battery will be needed to test this parameter, once it has been demonstrated that can have an important role in metal toxicity.

3.4.6. Global model

In the global model we integrated the essence of the interactions, and rejected parameters of less importance. The ultimate aim of the model was to find the simplest model that can account for significant influences on Daphnia's feeding rates, including just these variables into the model. We have to recognize that no model contains all the aspects of reality and our model, also,

will simplify the influence of certain water parameters on chronic effects to *D. magna*.

The experiments gave enough detail to estimate values for critical parameters chosen to characterize the feeding rate of *D. magna* (Straus) clone F at a specific body length: F_{max}, F_{half} and F_{index}.

The predictions, shown in Figure 3.4, plotted against the obtained feeding rates demonstrated that the predictions tend to sub-estimate the highest feeding rates obtained. For high values of feeding rates the model seemed to fail. Once the highest values of feeding rates occur for the biggest daphnids performed at 20º and 25º C, at constant food concentration, it can be discussed that for big daphnids, the model under-predicts the interaction between temperature and body length. This, in a first instance, can occur due to low adjustments of the Feeding coefficient and Feeding index predicted to 20º and 25º C.

This aspect is the biggest failure of the model because when analysing the residuals (feeding rates obtained less expected) we did not identify any tendency in over or under-estimation of the feeding rates according to food concentration, body length, temperature or medium (Figure 3.5).

Quantification of feeding rates under high food concentrations is technically difficult with the spectrophotometric methology since it would be necessary to use longer exposures and/or a larger number of organisms per test vessel. Either of this approach would necessarily introduce more bias in the results making it more difficult to interpret the results.

3.5. Conclusions

The presented model provides a general tool to simulate the feeding rates of *D. magna,* Clone F, at the individual level under varying body length, temperature, food concentration, hardness and alkalinity. Such predictive model for feeding rates can predict this endpoint along life-cycle of the organisms.

We showed that the biggest factor affecting organisms at a certain body length is the temperature, once an increase of temperature to 30º C results in lower feeding rates which, ultimately, lead to reduced life-cycles of the organisms.

The results obtained in this study are important both in terms of the effects of natural water conditions in the feeding rates of *D. magna* and in terms of toxicity tests. The reduction of the feeding rate of *D. magna* according to certain environmental conditions could result in great changes at the population levels, once growth and survival are dependent of the amount of energy (obtained by food) intake.

Future research

At this point, we still did not well characterize the physic and chemical variation in field situations. We need to test the influence of low temperatures and test pH under acidic waters.

3.6. References

Alstad, N.E.W., Skardal, L., Hessen, D.O., 1999. The Effect of Calcium Concentration on the Calcification of *Daphnia magna*. Limnol. Oceanogr. 44.

Bianchini, A., Martins, S.E.G., Barcarolli, I.F., 2005. Mechanism of acute copper toxicity in euryhaline crustaceans: implications for the Biotic Ligand Model. Animals and Environment 1275, 189-194.

Boeckman, C., Bidwell, J., 2006. The Effects of Temperature, Suspended Solids, and Organic Carbon on Copper Toxicity to Two Aquatic Invertebrates. Water Air Soil Poll. 171, 185-202.

Chakoumakos, C., Russo, R.C., Thurston, R.V., 1979. Toxicity of copper to cutthroat trout (*Salmo clarki*) under different conditions of alkalinity, pH and hardness. Environ. Sci. Technol. 13, 213-219.

de Mott, W.M., 1982. Feeding selectivities and relative ingestion rates of *Daphnia* and *Bosmina*. Limnol. Oceanogr. 27, 518-527.

de Schamphelaere, K.A.C., Janssen, C.R., 2002. A Biotic Ligand Model Predicting Acute Copper Toxicity for *Daphnia magna*: The Effects of Calcium, Magnesium, Sodium, Potassium, and pH. Environ. Sci. Technol. 36, 48-54.

Di Toro, D.M., Allen, H.E., Bergman, H.L., Meyer, J.S., Paquin, P.R., Santore, R.C., 2001. Biotic ligand model of the acute toxicity of metals. 1. Technical basis. Environ. Toxicol. Chem. 20, 2383-2396.

Gauld, D.T., 1951. The grazing rate of planktonic copepods. J. Mar. Biol. Assoc. Uk. 29, 695-706.

Gauthier, C., Couture, P., Pyle, G.G., 2006. Metal effects on fathead minnows (*Pimephales promelas*) under field and laboratory conditions. Ecotox. Environ. Safe. 63, 353-364.

Gliwicz, Z., Guisande, C., 1992. Family planning in *Daphnia*: resistance to starvation in offspring born to mothers grown at different food levels. Oecologia 91, 463-467.

Glover, C.N., Wood, C.M., 2005. Physiological characterisation of a pH- and calcium-dependent sodium uptake mechanism in the freshwater crustacean, *Daphnia magna*. The Journal of Experimental Biology 208, 951-959.

Goss, L.B., Bunting, D.L., 1980. Temperature effect on zooplankton respiration. Comp. Biochem. Phys. A 66, 651-658.

Gurney, W.S.C., McCauley, E., Nisbet, R.M., Murdoch, W.W., 1990. The Physiological Ecology of Daphnia: Development of a Model of Growth and Reproduction. Ecological Society of America 71, 716-732.

Haefner, J.W., 2005. Modeling Biological Systems: Principles and Applications. Springer Science + Business Media, Inc., USA.

Hallam, T.G., Lassiter, R.R., Li, J., Suarez, L.A., 1990. Modelling Individuals Employing an Integrated Energy Response: Application to *Daphnia*. Ecology 71, 938-954.

Heijerick, D.G., De Schamphelaere, K.A.C., Van Sprang, P.A., Janssen, C.R., 2005. Development of a chronic zinc biotic ligand model for *Daphnia magna*. Ecotox. Environ. Safe. 62, 1-10.

Hessen, D.O., Alstad, N.E.W., Skardal, L., 2000. Calcium limitation in *Daphnia magna*. J. Plankton Res. 22, 553-568.

Heugens, E.H.W., Hendriks, A.J., Dekker, T., van Straalen, N.M., Admiraal, W., 2002. A Review of the Effects of Multiple Stressors on Aquatic Organisms and Analysis of Uncertainty Factors for Use in Risk Assessment. Crit. Rev. Toxicol. 31, 247 - 284.

Jak, R.G., J.L., M., M.C.T., S., 1996. Evaluation of laboratory derived toxic effect concentrations of a mixture of metals by testing fresh-water plankton communities in enclosures. Water Res. 30, 1215–1227.

Kersting, K., 1978. Some features of feeding, respiration and energy conversion of *Daphnia magna*. Hydrobiologia 59, 113-120.

Kersting, K., van der Leeuw, W., 1976. The use of the coulter counter for measuring the feeding rates of *Daphnia magna*. Hydrobiologia 49, 233-237.

Kirk, K.L., 1991. Inorganic Particles Alter Competition in Grazing Plankton: The Role of Selective Feeding. Ecology 72, 915-923.

Lye Koh, H., Hallam, T.G., Ling Lee, H., 1997. Combined effects of environmental and chemical stressors on a model *Daphnia* population. Ecol. Model. 103, 19-32.

Mckee, D., Ebert, D., 1996. The interactive effects of temperature, food level and maternal phenotype on offspring size in *Daphnia magna*. Oecologia 107, 189-196.

McMahon, J.W., 1965. Some physical factors influencing the feeding behavior of *Daphnia magna* Straus. Can. J. Zool. 43, 603-611.

McWilliam, R.A., Baird, D.J., 2002. Postexposure feeding depression: a new toxicity endpoint for use in laboratory studies with *Daphnia magna*. Environ. Toxicol. Chem. 21, 1198-1205.

Nandini, S., Picazo-Paez, E.A., Sarma, S.S.S., 2007. The combined effects of heavy metals (copper and zinc), temperature and food (*Chlorella vulgaris*) level on the demographic characters of *Moina macrocopa* (Crustacea: Cladocera). J Environ Sci Health A Tox Hazard Subst Environ Eng 42, 1433-1442.

Nogueira, A.J.A., Baird, D.J., Soares, A.M.V.M. Testing Physiologically-based resource allocation rules in laboratory experiments with *Daphnia magna* Straus. Ann. Limnol. - Int. J. Lim. 40(4), 257-267.

OECD, 2004. OECD Guidelines for the Testing of Chemicals. Guideline 202: *Daphnia* sp., Acute Immobilisation Test, adopted April 2004.

Orchard, S.J., Holdway, D.A., Barata, C., Dam, R.A.V., 2000. A Rapid Response Toxicity Test Based on the Feeding Rate of the Tropical Cladoceran *Moinodaphnia macleayi*. Ecotox. Environ. Safe. 53, 12-19.

Perrin, N., 1988. Why are offsprings born larger when it is colder? Phenotypic plasticity for offspring size in the cladoceran *Simocephalus vetulus* (Müller). Funct. Ecol. 2, 283-288.

Plath, K., 1998. Adaptive feeding behavior of *Daphnia magna* in response to short-term starvation. Limnol. Oceanogr. 43, 593-599.

Playle, R.C., 1998. Modelling metal interactions at fish gills. Sci. Total. Environ. 219, 147-163.

Porter, K.G., Gerritsen, J., Jr., J.D.O., 1982. The effect of food concentration on swimming patterns, feeding behaviour, ingestion, assimilation, and respiration by *Daphnia*. Limnol. Oceanogr. 27, 273-288.

Pörtner, H.O., 2001. Climate change and temperature-dependent biogeography: oxygen limitation of thermal tolerance in animals. Naturwissenschaften 88, 137-146.

Santore, R.C., Mathew, R., Paquin, P.R., DiToro, D., 2002. Application of the biotic ligand model to predicting zinc toxicity to rainbow trout, fathead minnow, and *Daphnia magna*. Comp. Biochem. Phys. C 133, 271-285.

Schindler, D.W., 1968. Feeding, Assimilation and Respiration Rates of *Daphnia magna* Under Various Environmental Conditions and their Relation to Production Estimates. The Journal of Animal Ecology 37, 369-385.

Vijverberg, J., 1976. The effect of food quantity and quality on the growth, birth-rate and longevity of *Daphnia hyalina* leydig. Hydrobiologia 51, 99-108.

Wærvagen, S.B., Rukke, N.A., Hessen, D.O., 2002. Calcium content of crustacean zooplankton and its potential role in species distribution. FreshWater Biol. 47, 1866-1878.

Weetman, D., Atkinson, D., 2004. Evaluation of alternative hypotheses to explain temperature-induced life history shifts in *Daphnia*. J. Plankton Res. 26, 107-116.

Zar, J. H., 1998. Biostatistical Analysis. Prentice Hall Inc., Englewood Cliffs. New Jersey.

4. General Discussion

The development of a Biotic Ligand Model to predict acute and chronic responses to copper and zinc heavy metals in freshwater environments requires the comprehension about how do these metals behave in water with distinct physical and chemical properties.

Copper (FairweatherTait 1997) and zinc (Caffrey and Keating 1997) are essential micronutrients to aquatic biota required at low concentrations, playing an important role in a number of important enzymes. However, these metals are also economically important to numerous anthropogenic activities resulting in frequent releases into aquatic ecosystems promoting toxic effects to the aquatic communities (Hogstrand, Verbost et al. 1996). At the individual level, the toxic effect is a result of reduced homeostatic control due to sodium (Rainbow 1997) and calcium (Hogstrand, Wilson et al. 1994) net losses, respectively to copper and zinc expositions. The severe net loss of these macronutrients results in death of the organisms (Hyne, Pablo et al. 2005). However, if organisms can resist to the initial shock period, then some physiological adjustments are done and organisms can live under chronic copper (Hansen, Lipton et al. 2002) and zinc (Muyssen, De Schamphelaere et al. 2006) exposure conditions .

The investigation conducted by several researchers demonstrated that copper seems to be more toxic for aquatic species than zinc, once lower copper concentrations are needed to exert the same harmful consequences to organisms. The major toxicity of copper comparing to zinc toxicity has been attributed to higher stability binding constants (log K) of copper to the biotic ligands (Heijerick, De Schamphelaere et al. 2002). In our review we also found that although the existence of considerable published data about acute copper and zinc toxicity under ranging environmental characteristics, chronic zinc and copper toxicity reports for both fish and crustaceans are scarce.

Major cation concentration has been demonstrated to influence metal's toxicity due to its competition for the active binding sites of aquatic organisms (Erickson, Benoit et al. 1996). They are poor competitors for the biotic ligands, once their stability constants are lower when compared to the metal-gill stability

constants, but once they are present at high concentrations they can effectively reduce the toxic effect by binding to the biotic ligands (Kim, Gu et al. 2001). Calcium and magnesium, the hardness ions, and sodium are important competitors of zinc and copper to the biotic ligands (Erickson, Benoit et al. 1996). However, it was suggested that their relative importance is not the same. The calcium cations seem to be the most important competitors present in the medium (Heijerick, De Schamphelaere et al. 2002).

Hardness was the first parameter identified and included in regulatory policies (USEPA 1993) and its protector effect to aquatic biota has been demonstrated. However its relative importance was questioned because it does not distinguish between calcium and potassium (the hardness cations) effects (Naddy, Stubblefield et al. 2002). Few authors demonstrated that different species were affected in a different way according to the hardness relative composition, i. e., different calcium or magnesium rates can result in different toxic responses for species (Perschbacher and Wurts 1999). But at a chronic level some authors demonstrated that hardness has little impact in protecting cladocerans against copper toxicity (de Schamphelaere and Janssen 2004) while zinc chronic expositions remain affected by hardness ions (Heijerick, Janssen et al. 2003). The competition of cations with the metal would be of lower importance because copper was present at lower concentrations and due to the longer exposition period that the organisms are exposed at a chronic level (de Schamphelaere and Janssen 2004).

Alkalinity increases, usually accompanied by pH increases, account to the reduction of metal toxicity at both acute and chronic level, once its increase reduces the free metal activity, considered the most toxic species (Gensemer, Naddy et al. 2002). This results in the increase of metal hydroxides, and also carbonates, that are less bioavailable to aquatic biota (Chakoumakos, Russo et al. 1979). However, this effect is of lower importance for zinc because, at circumneutral pH values, the speciation of zinc is dominated by the free hydrated metal ion (Barata, Baird et al. 1998).

The pH has important implications in metal speciation (Stouthart, Haans et al. 1996) and, hence, in protection of fish and crustaceans against metal exposures. An increase in pH values results in an increase in hydroxide and

carbonate species with binding properties to the free metal species (Stouthart, Haans et al. 1996). These metal species exert lower toxicity when compared to the free metal ion (Chakoumakos, Russo et al. 1979). However, in the copper toxicity it seems that these species can explain some remaining toxicity that is not explained by the free metal activity (de Schamphelaere and Janssen 2002). Low pH values can, also, protect aquatic organisms exposed to acute toxicities due to the competing effect of hydrogen for the active sites of the organism. Hydrogen concentrations of (~10^{-5} M) have been suggested to significantly reduce metal toxicity (Long, Van Genderen et al. 2004). This competition effect is not so clear at chronic expositions (de Schamphelaere and Janssen 2004) .

The importance of natural organic matter is generally recognized (Dwane and Tipping 1998). Natural organic matter, usually quantified as the dissolved organic fraction, has been described as the greatest factor influencing heavy metal toxicity under acute and chronic expositions (de Schamphelaere and Janssen 2002). DOC concentration is the most frequent factor in determining the bioavailability of both copper and zinc, once differences in DOC concentration can lead to toxicities that vary several orders of magnitude (Van Genderen, Gensemer et al. 2007). However, the protective effect of DOC can be reduced with hardness increases because the organic matter ligands at higher hardness values can be occupied by the hardness cations, resulting in more free metal available to exert toxicity in the biotic ligands of the organisms (Breault, Colman et al. 1996). DOC concentrations of 5mg/L have been suggested to reduce the toxic effect for copper (Hollis, Burnison et al. 1996), while for zinc 10mg/L of DOC have been identified to ensure protection against zinc (Hyne, Pablo et al. 2005).

Some researchers, on the other hand, proposed that certain dissolved matter-metal complexes can be available at the active sites due to possible breaks in the active sites, resulting in additional toxic effects (Meems, Steinberg et al. 2004). They explained it as possible higher affinities (expressed by higher binding strength values) of the dissolved matter to the active sites, resulting in liberated free metal ion. DOC concentration *per si* cannot be a reliable indicator of the influence DOC. Some studies demonstrated that DOC source is an additional parameter that should to be considered, once DOC composition

varies from source to source and it can result in different toxicities due to different binding strength of their constituents (Ryan, Van Genderen et al. 2004). However, this aspect has been shown to be of greater importance for copper than for zinc, possibly due to the larger amounts of DOC required exerting the same inhibitory effects for zinc (Cheng and Allen 2006).

Our review demonstrated that the influence of natural organic matter will not possibly be completely explained by the inclusion of the dissolved fraction. The particulate organic fraction should be also included, once this component can be either a source or sink of metal toxicity (Hart 1982). This component is even of greater importance at chronic tests once organisms' food, usually phytoplankton, is a component of the particulate matter (Orchard, Holdway et al. 2000).

The particulate organic fraction can also be an important parameter of the medium and can protect or induce the metal toxicity (Boeckman and Bidwell 2006). Its influence has been neglected in the developed BLM and only the dissolved fraction has been identified, suggesting that heavy metals bound to the particulate fraction are not reactive (Santore, Mathew et al. 2002). However, some studies demonstrated that such assumption can lead to inaccuracies and the complexes metal-particle (such as algae, sand, peat and clay) can be partially available to the active sites of the organisms. Moreover, the presence of particulate matter can lead to toxic effects in the organisms (Erickson, Benoit et al. 1996). However, there will be necessary much more investigation in order to accurately understand the role of the particulate matter but the multiple composition of the particulate matter makes this task very hard to obtain.

The temperature influences the toxicity of metals because organisms usually have a range of favorable temperatures and outside the limits of such range the toxic effect of a metal is increased (Boeckman and Bidwell 2006).

The complexity of natural systems results in the inability of any one water quality parameter to be the single best predictor of risk assessment of these metals. While the single effect of the parameters reported above was identified to synthetic waters, in experiments conducted with natural waters, the effects of some of these parameters could obscure the effects of some others parameters influencing metal toxicity.

Finally, protective environmental policies have been designed to protect against chronic toxicity, not just acute toxicity. So, there is an urgent need for more research in terms of chronic response, especially for zinc toxicity, in order to provide protection to the aquatic fauna Water Framework Directive-WFD (Directive 2000/60/EC of the European Parliament).

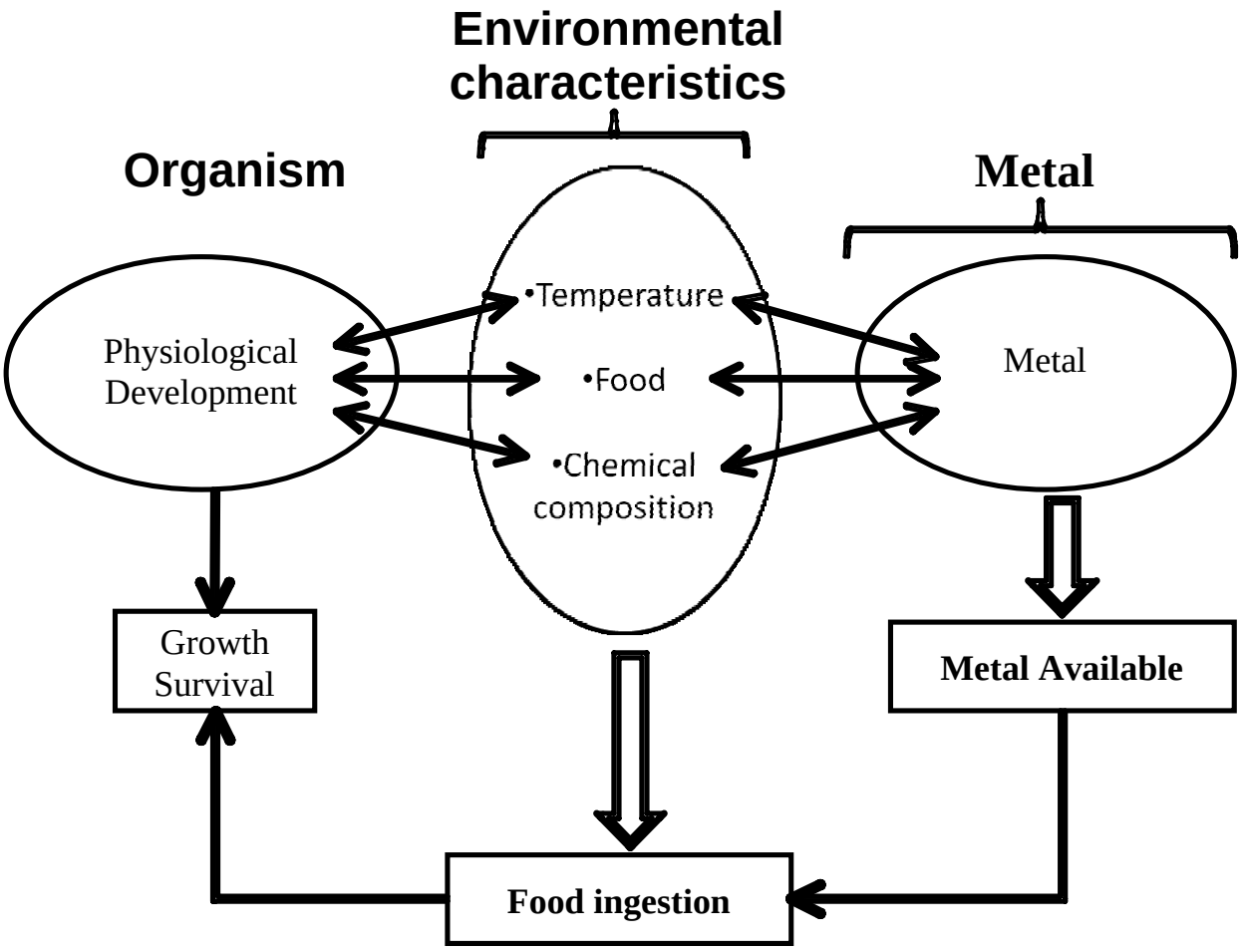

Figure 4.1 – Schematic diagram of the proposed BLM approach

The identification of these main water characteristics and their influence on metal toxicity was our starting point to the feeding assays aiming to develop a feeding model to *Daphnia magna* that, posterior, will be inserted in the Biotic Ligand Model for *Daphnia magna*, clone F (figure 4.1). The traditional acute and chronic tests do not reveal of how does a toxicant act. It has been suggested that the effects on the intrinsic rate of the population, obtained by reductions in reproduction, can be due two factors: reduction in food intake or increase in

metabolic costs (Jak, J.L. et al. 1996). The importance of measuring the feeding ingestion of the organism is, hence, of major importance in risk assessment of metals (McWilliam and Baird 2002). Consequently, our BLM approach will incorporate the feeding ingestion of the organisms and the temperature influence will not be neglected.

Our experimental design focused in variations of the physic and chemical water characteristics without metal exposition and their impacts in the organism's physiology because few reports exist about how do variations in major cations, alkalinity, pH and temperature influence the physiology of the organisms under their life cycle. The obtained data, using *Daphnia magna* demonstrated that the organisms had increased food requirements along the life cycle due to increased costs of maintenance with the increase of body length and reproductive costs (Gurney, McCauley et al. 1990). The feeding ingestion increased with the body length, following an allometric function as it was previously suggested by (Gurney, McCauley et al. 1990). Moreover, the organisms responded positively to the increased amounts of food present in the water. However, chemical variations of the tested media did not result in significant variations in feeding rates. Hardness and alkalinity individual variations did not result in significant variations in the amount of swallowed food as well as pH differences resulting from media composition variations. It was suggested that the tested chemical composition did not negatively affect the physiological development, at least for the feeding parameter. The amounts of macronutrients present at the media, ranging from low to very high alkalinities and from soft to very hard waters, were enough to the normal physiological development of the organisms. On the other hand, temperature increases resulted in significant feeding rates alterations, as it was reported by several authors (Mckee and Ebert 1996). Experiments conducted with organisms tested at 20º and 25º C suggested similar values of feeding rates, with feeding rates slightly higher at 25º C. When organisms were exposed to 30º C, the feeding rates decreased. The high metabolic rates addicted to lower feeding ingestions resulted in stress to the organisms (Weetman and Atkinson 2004). At higher temperatures, the process of molt is accelerated, organisms molt more often

requiring high energy to the metabolic processes which is obtained from food sources (Mckee and Ebert 1996). The result was smaller organisms with shorter life cycles (Goss and Bunting 1980). The feeding rates obtained at the three tested temperatures were, then, adjusted to a maximum function which described the feeding rates between the range of 20º and 30º C. This function determined that maximum feeding rates would be obtained near 24º C, which is in accordance with the literature (McMahon 1965).

5. Conclusions

Copper and zinc toxic effect in freshwater varies several orders of magnitude according to their concentration and bioa7vailability. The toxic effect has been demonstrated to be, essentially, due to the free metal ion activity, despite some other copper species have been suggested to exert additional toxic effects. At an acute level, the competitive effect of the cations, such as calcium, magnesium and sodium is of great importance. Although binding weakly to the active sites of the organisms, these cations, when present at high concentrations, compete with the free metal ion to the biotic ligands reducing the linkage of the free ion metal to the biotic ligand. At chronic exposures, however, their competitive effects seem to be of lower importance for copper, but more research is needed to determine their effects because few studies are available about this thematic.

Alkalinity and pH are important environmental parameters once they play an important role in metal speciation. Increases in alkalinity and pH result in increased amounts of carbonates and hydroxides, which bind free metal ions. At an acute level, the hydrogen ions exert an extra competitive effect to the binding sites when present at high concentrations (~10^{-5}M).

Organism's toxicity is also influenced by the environmental temperature and outside certain limits their capacity to exist is diminished. This factor, joint with metal exposition results in severe perturbations to the organisms.

Feeding assays were conducted using *Daphnia magna* along the life cycle was tested over independent hardness and alkalinity ranges and

temperatures that varied from 20º to 30º C. Data were adjusted to a general model predicting the feeding rates per organism where the significant factors altering the feeding rates were included. The feeding rates of the organisms followed an allometric function according to their body length. Moreover, daphnids significantly responded to the food level and to the temperature. The obtained curve for temperature registered an optimal temperature to *Daphnia magna*, clone F, at 24º C. Oppositely, organisms did not respond to chemical variations of medium

6. References

Barata, C., D. J. Baird, et al. (1998). "Influence of genetic and environmental factors on the tolerance of *Daphnia magna* Straus to essential and non-essential metals." Aquatic Toxicology 42(2): 115-137.

Boeckman, C. and J. Bidwell (2006). "The Effects of Temperature, Suspended Solids, and Organic Carbon on Copper Toxicity to Two Aquatic Invertebrates." Water, Air, & Soil Pollution 171(1): 185-202.

Breault, R. F., J. A. Colman, et al. (1996). "Copper Speciation and Binding by Organic Matter in Copper-Contaminated Streamwater." Environ. Sci. Technol. 30(12): 3477-3486.

Caffrey, P. B. and K. I. Keating (1997). "Results of Zinc deprivation in daphnid culture." Environmental Toxicology and Chemistry 16(3): 572-575.

Chakoumakos, C., R. C. Russo, et al. (1979). "Toxicity of copper to cutthroat trout (*Salmo clarki*) under different conditions of alkalinity, pH and hardness." Environmental Science & Technology 13(2): 213-219.

Cheng, T. and H. E. Allen (2006). "Comparison of zinc complexation properties of dissolved natural organic matter from different surface waters." Journal of Environmental Management 80(3): 222-229.

de Schamphelaere, K. A. C. and C. R. Janssen (2002). "A Biotic Ligand Model Predicting Acute Copper Toxicity for *Daphnia magna*: The Effects of

Calcium, Magnesium, Sodium, Potassium, and pH." Environ. Sci. Technol. 36(1): 48-54.

de Schamphelaere, K. A. C. and C. R. Janssen (2004). "Bioavailability and Chronic Toxicity of Zinc to Juvenile Rainbow Trout (*Oncorhynchus mykiss*): Comparison with Other Fish Species and Development of a Biotic Ligand Model." Environ. Sci. Technol. 38(23): 6201-6209.

Dwane, G. C. and E. Tipping (1998). "Testing a humic speciation model by titration of copper-amended natural waters." Environment International 24(5-6): 609-616.

Erickson, R. J., D. A. Benoit, et al. (1996). "The effects of water chemistry on the toxicity of copper to fathead minnows." Environmental Toxicology and Chemistry 15(2): 181-193.

FairweatherTait, S. J. (1997). "Bioavailability of copper." EUROPEAN JOURNAL OF CLINICAL NUTRITION 51: S24-S26.

Gensemer, R. W., R. B. Naddy, et al. (2002). "Evaluating the role of ion composition on the toxicity of copper to *Ceriodaphnia dubia* in very hard waters." Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 133(1-2): 87-97.

Goss, L. B. and D. L. Bunting (1980). "Temperature effect on zooplankton respiration." Comparative Biochemistry and Physiology A - Molecular and Integrative Physiology 66(A): 651-658.

Gurney, W. S. C., E. McCauley, et al. (1990). "The Physiological Ecology of *Daphnia*: Development of a Model of Growth and Reproduction." Ecological Society of America 71(2): 716-732.

Hansen, J. A., J. Lipton, et al. (2002). "Relationship between exposure duration, tissue residues, growth, and mortality in rainbow trout (*Oncorhynchus mykiss*) juveniles sub-chronically exposed to copper." Aquatic Toxicology 58(3-4): 175-188.

Hart, B. (1982). "Uptake of trace metals by sediments and suspended particulates: a review." Hydrobiologia 91-92(1): 299-313.

Heijerick, D. G., K. A. C. De Schamphelaere, et al. (2002). "Prediting acute zinc toxicity for *Daphnia magna* as a function of key water chemistry characterisitics: development and validation of a biotic ligand model." Environmental Toxicology and Chemistry 21(6): 1309-1315.

Heijerick, D. G., C. R. Janssen, et al. (2003). "The Combined Effects of Hardness, pH, and Dissolved Organic Carbon on the Chronic Toxicity of Zn to D. magna: Development of a Surface Response Model." Archives of Environmental Contamination and Toxicology 44(2): 210-217.

Hogstrand, C., P. M. Verbost, et al. (1996). "Mechanisms of zinc uptake in gills of freshwater rainbow trout: interplay with calcium transport." Am J Physiol Regul Integr Comp Physiol 270(5): R1141-1147.

Hogstrand, C., R. W. Wilson, et al. (1994). "Effects of zinc on the kinetics of branchial calcium uptake in freshwater rainbow trout during adaptation to waterborne zinc." J Exp Biol 186(1): 55-73.

Hollis, L., K. Burnison, et al. (1996). "Does the age of metal-dissolved organic carbon complexes influence binding of metals to fish gills?" Aquatic Toxicology 35(3-4): 253-264.

Hyne, R. V., F. Pablo, et al. (2005). "Influence of water chemistry on the acute toxicity of copper and zinc to the cladoceran *Ceriodaphnia dubia*." Environmental Toxicology and Chemistry 24(7): 1667-1675.

Jak, R. G., M. J.L., et al. (1996). "Evaluation of laboratory derived toxic effect concentrations of a mixture of metals by testing fresh-water plankton communities in enclosures." Water Research 30: 1215–1227.

Kim, S. D., M. B. Gu, et al. (2001). "Physicochemical Factors Affecting the Sensitivity of *Ceriodaphnia dubia* to Copper." Environmental Monitoring and Assessment 70(1): 105-116.

Long, K. E., E. J. Van Genderen, et al. (2004). "The effects of low hardness and pH on copper toxicity to *Daphnia magna*." Environmental Toxicology and Chemistry 23(1): 72-75.

Mckee, D. and D. Ebert (1996). "The interactive effects of temperature, food level and maternal phenotype on offspring size in *Daphnia magna*." Oecologia 107: 189-196.

McMahon, J. W. (1965). "Some physical factors influencing the feeding behavior of *Daphnia magna* Straus." Can. J. Zool. 43: 603-611.

McWilliam, R. A. and D. J. Baird (2002). "Postexposure feeding depression: a new toxicity endpoint for use in laboratory studies with *Daphnia magna*." Environmental Toxicology and Chemistry 21(6): 1198-1205.

Meems, N., C. E. W. Steinberg, et al. (2004). "Direct and interacting toxicological effects on the waterflea (*Daphnia magna*) by natural organic matter, synthetic humic substances and cypermethrin." Science of The Total Environment 319(1-3): 123-136.

Muyssen, B. T. A., K. A. C. De Schamphelaere, et al. (2006). "Mechanisms of chronic waterborne Zn toxicity in *Daphnia magna*." Aquatic Toxicology 77(4): 393-401.

Naddy, R. B., W. A. Stubblefield, et al. (2002). "The effect of calcium and magnesium ratios on the toxicity of copper to five aquatic species in freshwater." Environmental Toxicology and Chemistry 21(2): 347-352.

Orchard, S. J., D. A. Holdway, et al. (2000). "A Rapid Response Toxicity Test Based on the Feeding Rate of the Tropical Cladoceran *Moinodaphnia macleayi*." Ecotoxicology and Environmental Safety 53: 12-19.

Perschbacher, P. W. and W. A. Wurts (1999). "Effects of calcium and magnesium hardness on acute copper toxicity to juvenile channel catfish, *Ictalurus punctatus*." Aquaculture 172(3-4): 275-280.

Rainbow, P. S. (1997). "Ecophysiology of Trace Metal Uptake in Crustaceans." Estuarine Coastal and Shelf Science 44(2): 169-175.

Ryan, A. C., E. J. Van Genderen, et al. (2004). "Influence of natural organic matter source on copper toxicity to larval fathead minnows (*Pimephales promelas*): implications for the biotic ligand model." Environmental Toxicology and Chemistry 23(6): 1567-1574.

Santore, R. C., R. Mathew, et al. (2002). "Application of the biotic ligand model to predicting zinc toxicity to rainbow trout, fathead minnow, and *Daphnia magna*." Comparative Biochemistry and Physiology C - Toxicology & Pharmacology 133(1-2): 271-285.

Stouthart, X., J. L. M. Haans, et al. (1996). "Effects of water pH on copper toxicity to early life stages of the common carp (*Cyprinus carpio*)." ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY 15(3): 376-383.

USEPA (1993). Science Advisory Board. Notification of Consultation on Revisions to the Aquatic LifeWater Quality Criteria Guidelines, EPA-SABEPEC-CON-93-006.

Van Genderen, E., R. Gensemer, et al. (2007). "Evaluation of the Biotic Ligand Model relative to other site-specific criteria derivation methods for copper in surface waters with elevated hardness." Aquatic Toxicology 84(2): 279-291.

Weetman, D. and D. Atkinson (2004). "Evaluation of alternative hypotheses to explain temperature-induced life history shifts in *Daphnia*." J. Plankton Res. 26(2): 107-116.